

E2F6 in Axial Skeletal Development and Gliosis

by

Laurie Beth Friesenhahn

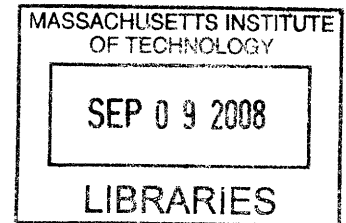
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ABSTRACT

E2F transcription factors were originally identified as regulators of cell cycle and cellular proliferation. *In vivo* mouse models have uncovered novel roles for these proteins in different developmental processes. This dissertation examines the biological role of E2F6 in mammalian development. E2F6 functions as a repressor of transcription in concert with the polycomb group (PcG) proteins and chromatin modifiers. PcG proteins regulate processes required for proper embryonic development and differentiation.

E2F6 interacts with core components of Polycomb Repressive Complex 1 (PRC1) and participates in PcG-mediated repression of *Hox* genes. *Hox* genes are required for correct patterning of the mammalian skeleton. Loss of *E2f6* results in posterior axial skeletal transformations. Mice deficient for both *E2f6* and *Bmi1*, a component of PRC1, exhibit increased penetrance of axial skeletal transformations. Thus, E2F6 and Bmi1 cooperate in the regulation of *Hox* genes and axial skeletal development. Bmi1 also represses transcription of the *Ink4a-Arf* locus, and it is consequently required to maintain the proliferative and self-renewal properties of hematopoietic and neural stem cells. However, E2F6 does not participate in the repression of the *Ink4a-Arf* locus. These findings underscore the significance of the E2F6-Bmi1 interaction *in vivo* and suggest that the *Hox* and *Ink4a-Arf* loci are regulated by somewhat different mechanisms.

In addition to axial skeletal transformations, *E2f6*^{-/-} mice exhibit a suppressed gliotic response after neural injury. Gliosis occurs in response to neurodegeneration, ischemia, and neuronal cell death. This process provides neuronal protection by restricting inflammation and regulating the concentration of molecules in the extracellular environment. However, gliosis has potentially detrimental effects such as the inhibition of axonal regeneration or the release of cytotoxic agents that trigger degeneration of neighboring neurons. The molecular mechanisms required to initiate and sustain a gliotic response are poorly understood. Gliosis is the focus of therapy for neurodegenerative diseases and ischemia, and complete understanding of the mechanisms underlying this process will lead to more effective therapies for neurodegenerative disease and ischemia.

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Chapter 1

Introduction

I. The E2F Family of Transcription Factors

The E2F transcription factors are classically described as key regulators of the cell cycle and cellular proliferation. The cellular targets of E2F include cell-cycle regulators; components of the DNA replication machinery; proteins involved in chromatin modification, assembly, condensation, segregation; and nucleotide biosynthesis (some classic E2F target genes are listed in Table 1). It is now clear that E2F also plays important roles in development and disease. Many studies have linked the amplification of E2Fs to various cancers (Oeggerli *et al.*, 2006; Orlic *et al.*, 2006; Hurst *et al.*, 2007). Further research has reported a requirement for different E2Fs in developmental processes such as heart development (Cloud *et al.*, 2002), axial skeletal development (Courel *et al.*, 2008), and embryonic development (Li *et al.*, 2008).

1. Discovery and cloning of E2F

E2F was first identified in research based upon the observation that the adenovirus E1A (early region 1A) protein stimulates the transcription of several viral and cellular promoters (Nevins, 1981). When Kovesdi *et al.* infected cells with E1A, they discovered that the induction of transcription by E1A was dependent upon a cellular factor, E2F (E2 factor) (Kovesdi *et al.*, 1986). The induction was determined to be independent of protein synthesis (Reichel *et al.*, 1988). Subsequent research found that E1A induces transcription by dissociating cellular E2F complexes. This dissociation releases free E2F and activates E2F's transcriptional activity (Bagchi *et al.*, 1990). Yee *et al.* investigated the nature of E2F's induction of the E2 promoter. DNase footprinting

Cell Cycle		
		References
Cdc2	Cell Division Cycle 2	(Tommasi and Pfeifer, 1995)
Cdc25	Cell Division Cycle25	(Stevaux and Dyson, 2002)
E2f1	E2 promoter binding factor 1	(DeGregori <i>et al.</i> , 1995a; Li <i>et al.</i> , 2008)
Cdk2	Cyclin dependent kinase 2	(Ren <i>et al.</i> , 2002; Stevaux and Dyson, 2002)
P107	Retinoblastoma-associated protein homolog 107	(Zhu <i>et al.</i> , 1995)
pRB	Retinoblastoma-associated protein	(Ren <i>et al.</i> , 2002)
CycE	Cyclin E	(Botz <i>et al.</i> , 1996) (Ohtani <i>et al.</i> , 1995; Geng <i>et al.</i> , 1996)
CycA	Cyclin A	(Schulze <i>et al.</i> , 1995)
B-myb		(Zwicker <i>et al.</i> , 1997)
C-myc		(DeGregori <i>et al.</i> , 1995a)
CycD	Cyclin D	(DeGregori <i>et al.</i> , 1995a)
Replication		
PolA	DNA polymerase α	(DeGregori <i>et al.</i> , 1995a)
Orc1	Origin recognition complex 1	(Ohtani <i>et al.</i> , 1996)
Mcm	Minichromosome maintenance	(Ren <i>et al.</i> , 2002)
Cdc6	Cell division cycle 6	(Lavia and Jansen-Durr, 1999)
PCNA	Proliferation cell nuclear antigen	(DeGregori <i>et al.</i> , 1995a)
TK1	Thymidine Kinase	(DeGregori <i>et al.</i> , 1995a)
TOP2A	Topoisomerase 2 α	(Ren <i>et al.</i> , 2002)
DHFR	Dihydrofolate reductase	(Means <i>et al.</i> , 1992; Wade <i>et al.</i> , 1992; Lavia and Jansen-Durr, 1999)

Table 1: Classic E2F Target Genes

and DNA mutagenesis studies revealed two distinct E2F binding sites that were required for transcription of the E2 protein (Yee *et al.*, 1987). The discovery of an E2F binding site allowed purification of E2F from crude extracts by chromatography and DNA purification schemes. This purification yielded a 54kD protein that could bind to the E2F consensus site and stimulate transcription in an *in vitro* reporter assay (Yee *et al.*, 1989).

In addition to E2Fs known role in the activation of viral genes, many scientists hypothesized that E2F may have a similar cellular function. Indeed, many genes involved in proliferation contain an E2F binding site, and E2F binding activity increases upon serum stimulation of cells (Mudryj *et al.*, 1990). This observation was the first step to realizing E2F's larger role in the control of proliferation and the cell cycle.

An important breakthrough in the elucidation of the mechanism of E2F's transcriptional transactivation was the discovery that E2F is a cellular target of the retinoblastoma protein, pRB (Chellappan *et al.*, 1991; Chittenden *et al.*, 1991; Kaelin *et al.*, 1991). pRB was known to control cell cycle and proliferation, but the mechanisms were unknown (Chittenden *et al.*, 1991; Kaelin *et al.*, 1991). pRB and two related proteins, p107 and p130, are a family of transcription factors known as the pocket proteins. The Rb-E2F interaction was the basis for cloning E2F. Helin and colleagues screened a λ expression library for cDNAs whose protein products had the ability to bind the pocket region of pRB. They characterized a cDNA and its protein product that interacts with pRB, binds to E2F recognition sequences, and transactivates the E2 promoter. Additionally, E1A disrupted the binding of this cDNA's protein and pRB (Helin *et al.*, 1992). This cDNA was ultimately determined to be the coding sequence of E2F. The cloning of E2F was a milestone because it led to the discovery of an entire family of E2F transcription factors that have the ability to either activate or repress transcription. The E2F transcription factors work in concert with the pocket proteins to control the entry and progression through the cell cycle.

2. The E2F/RB pathway

The pocket proteins are named based on their pocket-like structure. This pocket is made of two sub-domains separated by a spacer (Ewen *et al.*, 1991). Although the pocket proteins have a similar structure, it is clear that they have different affinities for different E2Fs. The activating E2Fs (E2F1, E2F2, and E2F3) associate almost exclusively with pRB, and the repressive E2Fs (E2F4 and E2F5) associate mainly with p107 and p130.

pRB is often referred to as the restriction point switch of the cell cycle. Indeed, the phosphorylation of pRB is a major determinant of whether the cell commits to a cell cycle or re-enters a resting state (G_0) (Bartek *et al.*, 1996). A cell's ability to surpass the restriction point when conditions for cell division are unfavorable is the basis for many cancers. For this reason, it is important to know the factors and signaling pathways that regulate this critical point in the cell cycle.

A resting cell in G_0 has low levels of pRB, low levels of the activating E2Fs, high levels of cyclin dependent kinase inhibitors (CDKIs), and high levels of p130 (Weinberg, 1995; Dyson, 1998). The predominant complexes are p130-E2F4 and p130/p107-E2F5 (Dyson, 1998). This concerted gene expression pattern results in suppression of S-phase and proliferation genes by two mechanisms. First, repressive pocket protein-E2F complexes allow for active repression by recruitment of chromatin modifiers and histone deacetylases that actively repress transcription. Second, pRB complexed with activating E2Fs renders them functionally inactive and leads to a loss of transcriptional activation of these genes (Figure 1) (Dyson, 1998; Trimarchi and Lees, 2002). The high levels of CDKIs also suppress cell cycle progression by inhibiting the activity of any cyclin/cdk complex that may be present (Dyson, 1998; Trimarchi and Lees, 2002).

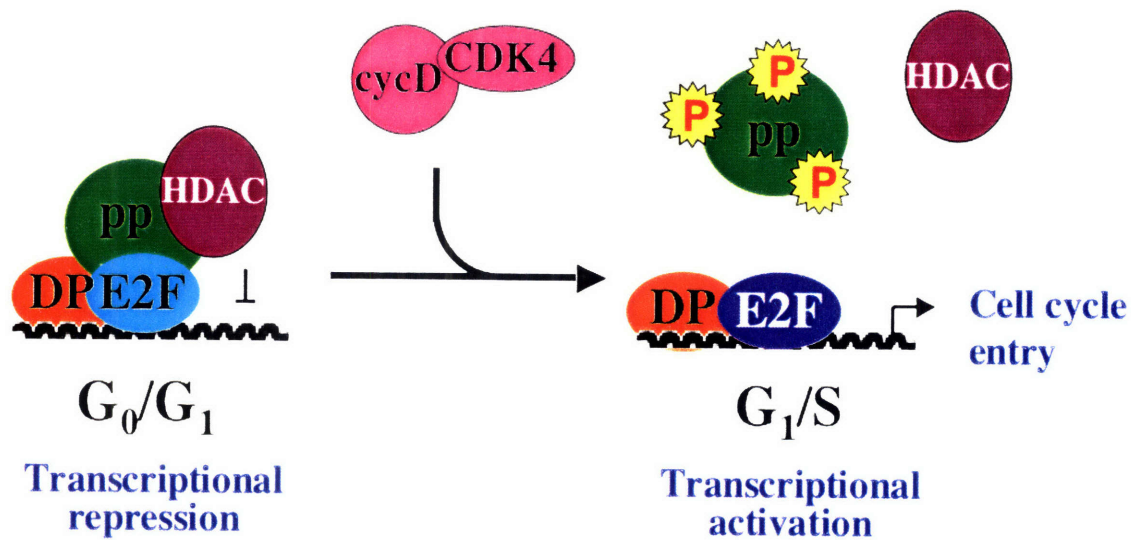


Figure 1: Transcriptional repression and activation by E2F.

E2F target genes are regulated in G_0/G_1 by a repressive complex consisting of repressive E2Fs/DP, histone deacetylases (HDAC), and pocket proteins (pp). Upon mitogenic stimulation, $cycD/CDK$ complexes phosphorylate the pocket proteins. This phosphorylation leads to the disruption of pocket protein-E2F complexes, causing the activating E2Fs to bind the promoters of cell cycle regulated target genes and activate their transcription.

Upon mitogenic stimulation, expression of cyclin D1 is induced (Matsushime *et al.*, 1991) and expression of CDKIs are reduced (Polyak *et al.*, 1994). This gene expression pattern shifts the CDKI-to-cyclin ratio and relieves inactivation of the cyclins by the CDKIs (Weinberg, 1995). Cyclin D forms an active complex with cdk4 or cdk6 and then phosphorylates pRB. Phosphorylation of pRB causes the dissociation of E2F-pRB complexes, and the activating E2Fs are now free to stimulate transcription of E2F-target genes (Figure 1) (Weinberg, 1995; Dyson, 1998; Mittnacht, 1998). This first wave of pRB phosphorylation and E2F transcriptional activity leads to increased transcription of Cyclin E, an E2F-target gene. Cyclin E and cdk2 form an active complex that hyperphosphorylates pRB, p107, and p130 (Weinberg, 1995; Dyson, 1998; Mittnacht, 1998). This results in disassembly of E2F-pocket protein complexes, a process that is essential for entry into S-phase. Free, activating E2Fs now initiate transcription of S-phase genes (Weinberg, 1995; Dyson, 1998; Mittnacht, 1998; Trimarchi and Lees, 2002). Conversely, E2F4 and E2F5 lack a nuclear localization signal, and dissociation from the pocket proteins results in their translocation from the nucleus and loss of transcriptional repression of S-phase genes (Muller *et al.*, 1997; Chestukhin *et al.*, 2002). Activation of gene expression, control of cyclin-cdk activity, and regulation of pRB phosphorylation are critical for a cell's entry and commitment to cell division.

3. Regulation, structural identity, and transcriptional activity of different E2Fs

The E2F transcription factors are essential for coordinated control of cell division and proliferation. For this reason, it is not surprising that deregulation of E2F is the hallmark of many cancers (Sherr, 1996). Understanding the regulation of E2F and the

mechanisms by which E2F confers control on the cell cycle is essential to studies in cancer and other diseases.

To date, nine E2F and two DP (DRTF1 polypeptide) proteins have been identified. DP is the heterodimerization partner of E2F transcription factors, and it is required for the DNA binding activity of E2Fs 1-6 (Bandara *et al.*, 1993; Huber *et al.*, 1993). The E2F-DP complex creates a basic helix-loop-helix transcription factor that recognizes the consensus DNA sequence 5'-TTTCGCGC-3' (Zheng *et al.*, 1999; Trimarchi and Lees, 2002). E2Fs 1-8 can be divided into subclasses based on their structure, expression pattern, ability to bind pocket proteins, and transcriptional activity (Figure 2). The E2Fs 1-6 share two structural domains: a DNA binding domain and a dimerization domain (Trimarchi and Lees, 2002). The dimerization domain is necessary for heterodimerization with a DP (DRTF1 polypeptide) subunit (Zheng *et al.*, 1999). This interaction is required for high affinity binding to E2F consensus sites (Slansky and Farnham, 1996). E2F7 and E2F8, the newest members of the E2F family, have two conserved DNA binding domains but lack a dimerization domain. Instead of heterodimerizing with DP, E2F7 and E2F8 can heterodimerize or homodimerize with themselves or with each other (Li *et al.*, 2008). Exogenous expression of E2F7 or E2F8 can repress E2F target genes and block cell proliferation (de Bruin *et al.*, 2003; Di Stefano *et al.*, 2003; Maiti *et al.*, 2005).

Similar to E2F7 and E2F8, E2Fs 4-6 are also active repressors of transcription of E2F-target genes (Muller *et al.*, 1997; Verona *et al.*, 1997; Trimarchi *et al.*, 1998; Giangrande *et al.*, 2004). E2F4, E2F5, and E2F6 have all been shown to interact with histone deacetylases, histone methylases, and other chromatin remodeling enzymes

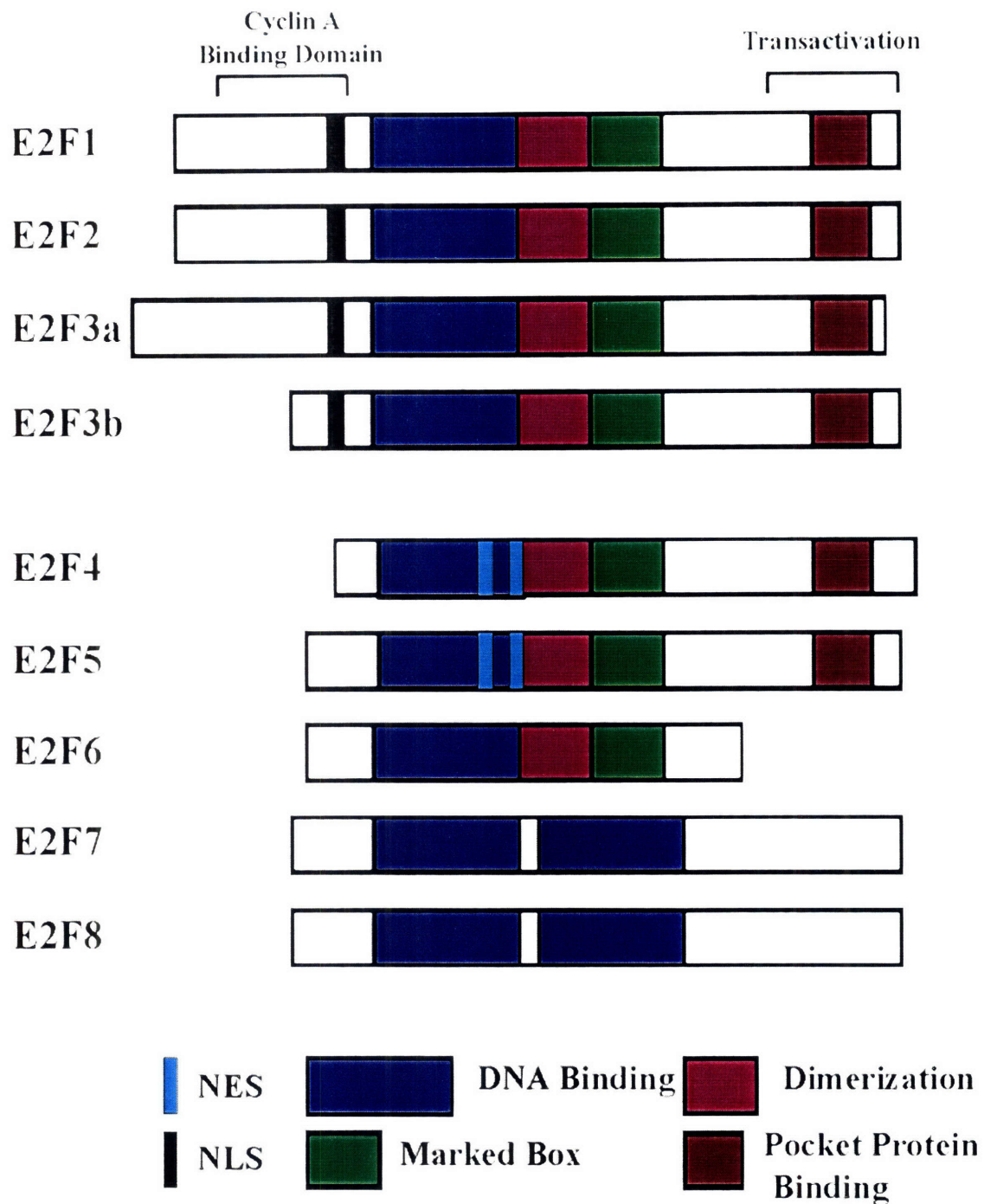


Figure 2: The E2F family of transcription factors.

The E2F transcription factors all contain a homologous DNA-binding region. The activating E2Fs, E2Fs 1-3, each contain a nuclear localization signal (NLS), a marked box, a dimerization region, and a pocket protein-binding region. The repressive E2Fs, E2Fs 4-5, lack a NLS but have a nuclear export signal (NES). E2Fs 6-8 lack sequences required for pocket protein binding and repress transcription through mechanisms independent of the pocket proteins.

associated with repressed chromatin (Iavarone and Massague, 1999; Ogawa *et al.*, 2002; Attwooll *et al.*, 2005). However, it is clear that E2F4 and E2F5 repress transcription in a mechanism that is different from E2F6. E2F4 and E2F5 contain a pocket protein binding domain and repress transcription through a pocket protein-E2F complex. Interaction of E2F4-DP and E2F5-DP is also induces the nuclear localization of these complexes (Trimarchi and Lees, 2002). E2F6 lacks a pocket protein binding domain and represses transcription through its association with the polycomb group family of transcription factors (discussed in more detail in following sections) (Trimarchi and Lees, 2002). E2F4 and E2F5 are present throughout the cell cycle (Slansky and Farnham, 1996), but they accumulate in quiescent cells (Ikeda *et al.*, 1996). In contrast, E2F6 levels increase as cells enter the cell cycle and peak at mid G1 (Dahme *et al.*, 2002). While the mechanisms of repression and expression patterns of these two sub-groups of E2Fs differ, their specific targets are overlapping, and it has been shown that E2F4 or E2F6 can compensate for the loss of the other (Giangrande *et al.*, 2004).

The last group of E2F transcription factors includes E2Fs 1-3. As with E2F4 and E2F5, these E2Fs also have a pocket protein binding domain, a dimerization domain, and a DNA binding domain (Helin, 1998; DeGregori, 2002; Trimarchi and Lees, 2002) (Dyson, 1998). However, E2Fs 1-3 are potent activators of transcription and their expression patterns are transcriptionally regulated throughout the cell cycle (Trimarchi and Lees, 2002). The transcription of these E2Fs is induced in late G1, and the gene products accumulate at this time (Slansky and Farnham, 1996). E2Fs 4-5 require the binding of a pocket protein to actively repress E2F-target genes. This repression is achieved through interactions with pocket proteins and histone deacetylases. However,

E2Fs 1-3 must be dissociated from a pocket protein before they become transcriptionally active (Sun *et al.*, 2007). Overexpression of the activating E2Fs can induce quiescent cells to re-enter the cell cycle and can override growth-arrest signals such as TGF β or cyclin dependent kinase inhibitors such as p16, p21, or p27 (DeGregori *et al.*, 1995a; DeGregori *et al.*, 1995b) (Schwarz *et al.*, 1995; Mann and Jones, 1996). It is clear that E2Fs 1-3 are potent activators of the cell cycle, and overexpression of an activating E2F can induce transformation of primary cells (Johnson *et al.*, 1994; Singh *et al.*, 1994; Xu *et al.*, 1995). Similarly, loss of E2F1, E2F2, and E2F3 leads to a decrease in E2F-target gene expression and a block in cell proliferation (Wu *et al.*, 2001).

Together, the E2Fs and pocket proteins are critical for a cell's entry and committal to cell division when conditions are favorable. The manner in which a cell responds to unfavorable signals and aborts the cell division program is equally important. When a cell undergoes DNA damage or oncogenic stress, a different program ensures that a damaged or potentially cancerous cell does not undergo division. At the backbone of this control are p19ARF and p53. These proteins are the basis of the ARF-p53 tumor surveillance network.

4. The Arf-p53 tumor surveillance network

p19^{ARF} is one of the proteins encoded in the INK4A-ARF locus. The other protein, p16^{INK4a}, is a CDKI and inhibits cyclin/cdk complexes and subsequently the G₁/S transition (Sherr and Roberts, 1999). p19^{ARF} is a tumor suppressor that responds to abnormal proliferative or oncogenic signals. Its activation leads to cell cycle arrest or apoptosis (Figure 3) (de Stanchina *et al.*, 1998; Palmero *et al.*, 1998; Radfar *et al.*, 1998). p19^{ARF} indirectly activates the tumor suppressor, p53, by binding and inhibiting Mdm2, a

repressor of p53 (Zhang *et al.*, 1998). Upon activation of p53 by p19^{ARF}, p53 increases the levels of p21 (a CDKI) or activates Bax proteins. This leads to growth arrest or apoptosis (Figure 3) (Lundberg and Weinberg, 1999).

The deregulation of INK4a-ARF or the E2F-RB pathway occurs in a significant fraction of cancers. This suggests that circumvention of this pathway leads to cellular transformation. Research of the complex mechanisms that control whether or not a cell divides is critical to understanding the process of tumorigenesis.

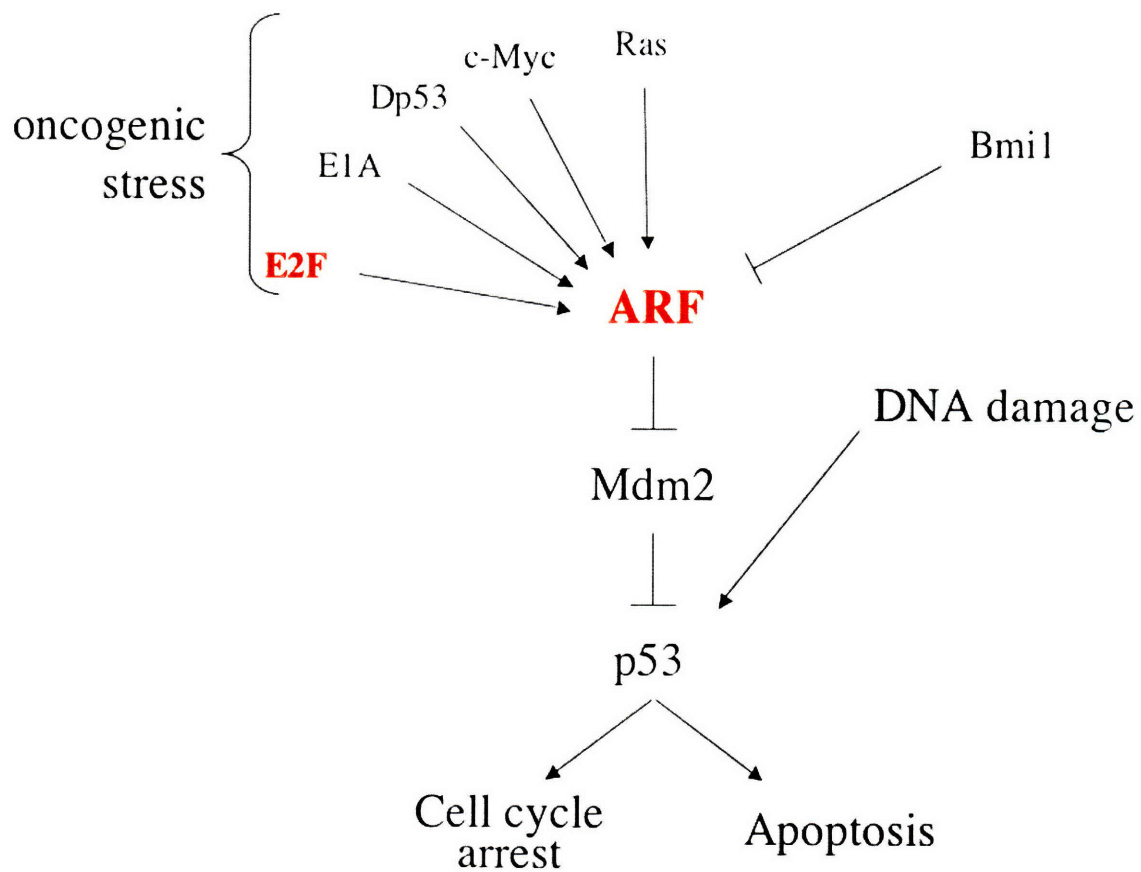


Figure 3: The Arf-p53 tumor surveillance pathway.

p19^{ARF} is activated by abnormal proliferative or oncogenic signals and is repressed in normal proliferating cells by a polycomb group protein, Bmi1. Activation of this pathway results in cell cycle arrest or apoptosis.

II. E2Fs in Development and Disease

The E2F transcription factors are key regulators of the cell cycle. However, *in vivo* studies of the E2Fs in mouse models have uncovered roles for these proteins beyond cell cycle control and proliferation. E2Fs1-8 have been deleted in mice, and analysis of these mice has revealed overlapping and distinct roles for E2Fs in development, differentiation, and disease.

1. The activating E2Fs: E2F1, E2F2, and E2F3

The activating E2Fs are critical for cellular proliferation. Deletion of *E2fs1-3* results in severely impaired proliferation and cell cycle kinetics in cultured cells (Wu *et al.*, 2001). Disruption of activating E2Fs in mouse models reveals specific and overlapping functions for these E2Fs. *E2f1* mutant mice are fully viable but exhibit testicular atrophy, exocrine abnormalities, and a spectrum of tissue-specific tumors (Yamasaki *et al.*, 1996). Interestingly, overexpression of *E2f1* in mice leads to tumor development as well, suggesting that E2F1 can act as a tumor suppressor and an oncogene *in vivo* (Pierce *et al.*, 1999). In contrast to *E2f1* mutant mice, *E2f3* mutant mice exhibit embryonic lethality in a pure genetic background and are born at one quarter the expected frequency in a mixed genetic background. Although *E2f3* mutant mice do not develop tumors, they exhibit severe growth retardation and die prematurely from congestive heart failure (Cloud *et al.*, 2002). Analysis of *E2f1;E2f3* compound mutant mice revealed overlapping roles for these proteins. These mice had increased embryonic lethality, growth retardation, and increased severity in testicular atrophy and congestive heart failure. However, the compound mutant mice had no alteration in tumor incidence, timing, or spectrum (Cloud *et al.*, 2002). Recently, *E2f3a* and *E2f3b*-specific mutant mice

have been developed. These mice are viable, and they have no histological abnormalities. This suggests that *E2f3a* and *E2f3b* have overlapping roles *in vivo*. *E2f1;E2f3b* mutant mice are viable, and they have no tissue defects. *E2f1;E2f3a* mutant mice die perinatally and exhibit a defect in cartilage development. (Danielian, Friesenhahn *et al.*, in press; Appendix A). These studies reveal distinct roles for E2F1 and E2F3 *in vivo*.

E2f2 mutant mice, like *E2f1*, are born at the expected frequency. However, they die prematurely from systemic autoimmune disease caused by enhanced T lymphocyte proliferation (Murga *et al.*, 2001) and hematopoietic defects (Li *et al.*, 2003). *E2f1;E2f2* mutant mice are viable, but diabetes causes a premature death. These mice exhibit a reduction of size and cellularity of lymphoid organs, the testes, and salivary gland (Iglesias *et al.*, 2004). In this case, the resulting diabetes is caused by the combination of the defect in hematopoiesis (caused primarily from *E2f2* deletion) and exocrine degradation (caused primarily by the *E2f1* deletion) (Li *et al.*, 2003). E2F1 and E2F2 have distinct roles *in vivo*, but combinatorial loss of these two genes results in a novel disease phenotype.

2. The repressing E2Fs: E2F4 and E2F5

E2F4 and E2F5 play similar roles in the repression of cell cycle genes. However, *in vivo* studies revealed that these proteins have distinct roles in different developmental programs. Mutation of *E2f4* in mice uncovered a novel role for E2F4 in the control of erythropoiesis. *E2f4*^{-/-} mice exhibited severe anemia, low red blood cell numbers, macrocytosis, and Howell-Jolly bodies (Humbert *et al.*, 2000). While the mature red blood cells were abnormal, there was no defect detected in the immature erythrocytes, suggesting that E2F4 is required for the late stages of erythrocyte maturation. These

mice have abnormal craniofacial development, abnormal development of the gut epithelium, neonatal lethality, and growth retardation starting as early as E13.5 (Humbert *et al.*, 2000; Rempel *et al.*, 2000). Neither the erythroid defect nor the resulting anemia is the cause of the neonatal lethality. Instead, a defect in the development of cilia in the nasal epithelium makes these mice unable to clear infectious agents, which results in increased susceptibility to bacterial infections (Humbert *et al.*, 2000; Danielian *et al.*, 2007). Similar to *E2f4* mutant mice, *E2f5* mutant mice have normal cell cycle kinetics. These mice appear to develop normally prior to weaning age. After weaning, *E2f5* mutant mice developed ataxia, ruffled coats, and dehydration. Most of these mice died at around six weeks of age due to hydrocephalus (excessive cerebrospinal fluid production) and intracerebral hemorrhage. Lindeman *et al.* subsequently determined that E2F5 is important for the regulation of secretion of cerebrospinal fluid (Lindeman *et al.*, 1998). While E2F4 or E2F5 can compensate for each other *in vitro* during the regulation of cell cycle genes (Lindeman *et al.*, 1998; Humbert *et al.*, 2000), *in vivo* studies uncovered novel roles for these proteins in different developmental pathways.

3. E2F6

Although E2F6 is a repressor of transcription, it lacks a pocket protein binding domain and represses transcription through association with polycomb group proteins (Trimarchi and Lees, 2002). E2F6 has been shown to interact biochemically with the polycomb group proteins: EZH2, Bmi1, Mel-18, RYBP, Ring1a, and Ring1b (Trimarchi *et al.*, 2001; Ogawa *et al.*, 2002; Attwooll *et al.*, 2005). The polycomb group proteins (discussed more in-depth in the next section) are repressors of homeobox (*Hox*) genes, which control the anterior-posterior patterning of the developing embryo. Mice deficient

for E2F6 exhibit posterior transformations of the axial skeletons. These include: (1) a T13-L1 transformation as evidenced by the absence or incomplete development of ribs normally present on the thirteenth thoracic vertebra; and (2) a L6-S1 conversion in which the iliac bones associate with the sixth lumbar vertebra instead of the first sacral vertebra. These mice survive to birth and live a normal life span. *In vitro* studies did not reveal any cell cycle or proliferation defects (Storre *et al.*, 2002; Courel *et al.*, 2008). Further analysis of *E2f6* mutant mice revealed a defect in spermatocyte development, but the defect was not severe enough to affect fertility (Storre *et al.*, 2002). Recently, unpublished data suggests that E2F6 plays a role in the gliosis response of the brain after neuronal damage (Chapter 3). *E2f6*-mutant mice exhibit a suppression of gliosis when they have brain damage induced by *Bmi1*-loss or injection of a neurotoxin (Chapter 3), but it is not clear how *E2f6* loss suppresses gliosis.

Beyond *Hox* gene regulation, little is known about which genes E2F6 controls. In an attempt to understand more about the biological role of E2F6, some laboratories have conducted microarrays or chromatin immunoprecipitation followed by CpG island microarrays (Chip-Chip) to study which genes E2F6 regulates on a genome-wide scale. Oberley *et al.* used Chip-Chip on human tumor cells and found that E2F6 regulates genes involved in tumor suppression and maintenance of chromatin structure (Oberley *et al.*, 2003). Two other laboratories conducted cDNA microarray experiments with wildtype and *E2f6* mutant mouse embryonic fibroblasts (MEFs). These studies revealed that E2F6 is important for the repression of some meiotic (*Smc1 β* and *STAG3*) (Storre *et al.*, 2005) and testes-specific (α -tubulin 3 and 7) genes (Pohlers *et al.*, 2005). Although E2F6 is widely expressed in most tissues, little is known about genes that E2F6 regulates *in vivo*.

While derepression of the testes-specific genes could explain the defect in spermatocyte development, the mechanism by which E2F6 regulates gliosis is still unknown.

4. E2F7 and E2F8

E2F7 and E2F8 are the most recent E2F family members to have been identified. Homologous recombination techniques have been utilized to conditionally mutate each of these genes in mice. *E2f7* or *E2f8* mutant mice are viable, and they display no abnormalities. The *E2f7;E2f8* compound mutant mice die as early as E11.5. These embryos exhibit vascular defects, multifocal hemorrhages, and widespread apoptosis. RNA analysis from these embryos revealed a substantial increase in the level of *E2f1* mRNA. In wildtype cells, E2F7 and E2F8 occupy the promoter of *E2f1* and repress transcription. The elevated level of E2F1 is due to a loss of direct repression by E2F7 and E2F8. The massive apoptosis seen in the compound mutant animals is due to E2F1 overexpression, p53 accumulation, and activation of the apoptotic response of the cell. Microarray analysis from MEFs made from these embryos indicated a deregulation of genes involved in stress responses to hypoxia, nutrient deprivation, and apoptosis (Li *et al.*, 2008). It is unclear whether deregulation of these genes is due to a direct loss of E2F7 and E2F8 transcriptional repression or whether it is due to an indirect consequence of the apoptotic program that is activated in these cells. It will be interesting to learn what tissue-specific abnormalities arise in these mice when *E2f7* and *E2f8* are acutely deleted in different tissues.

5. Specificity of E2Fs in Development

It is clear the E2Fs have distinct and overlapping roles in development. While this is not surprising, it still raises the question of where the specificity arises among different

functional groups of E2Fs. There is some evidence to suggest that specificity arises from the spatial and temporal regulation of individual E2F family members. For example, E2Fs 4 and 5 exhibit disparate embryonic expression patterns. E2F4 is expressed early in proliferating cells and the epithelium, while E2F5 is mainly expressed in terminally differentiating or differentiated cells (Lindeman *et al.*, 1998; Humbert *et al.*, 2000; Rempel *et al.*, 2000). In fact, most E2Fs exhibit tissue and cell-type specific expression patterns during development (Dagnino *et al.*, 1997). This implies that, while E2Fs in the same functional group may be able to compensate for one another, diverse expression patterns may prevent them from doing so in certain tissues. Another theory is that different combinatorial interactions achieve the specificity of function seen in developmental processes. For example, in a yeast two-hybrid screen for E2F3-specific interacting partners, TFE3 was found to bind specifically to E2F3 but not other activating E2Fs. The E2F3-TFE3 complex synergistically controls transcription of the p68 gene (Giangrande *et al.*, 2003). It is likely that E2F specificity is achieved by a combination of these two mechanisms.

III. Regulation of *Hox* Genes by Polycomb Group Proteins.

Polycomb group proteins were first characterized in metazoan development as regulators of segment identity. A central question in this field was how embryonic cells first acquired and subsequently maintained unique positional identities. Through genetic analysis and mutational studies in *Drosophila*, the answer came with the discovery of Polycomb group (PcG) genes and their transcriptional targets, the homeotic genes (*Hom*). These genes were determined to be important regulators of cellular identity in *Drosophila*. The PcG genes and *Hom* genes (called *Hox* in vertebrate systems) are highly conserved in mammals, and these genes play a similar functional role. It is clear that PcG genes have roles beyond their transcriptional regulation of the *Hox* genes. Mutations of PcG proteins in mammalian systems lead to deregulation of *Hox* genes and patterning defects as well as other developmental abnormalities.

1. The Regulation of *Hom* genes in *Drosophila*

The *Drosophila* embryo is composed of segmented units, or a repeated pattern of elements along the anterior-posterior axis. This metameric organization is established within the first two hours following fertilization. This process requires coordinated expression of two groups of genes: the segmentation genes and the homeotic genes (*Hom*) (Akam, 1987). The segmentation genes are the first detectable zygotically active genes and are divided into three classes based on their mutated phenotypes. The first group consists of gap genes whose mutations cause multiple adjacent segments to be missing from the embryo (Nusslein-Volhard and Wieschaus, 1980; Qian *et al.*, 1993; Shimell *et al.*, 1994). The second class of genes includes the pair-rule genes whose

mutations cause alternate segment-size units to be missing (Nusslein-Volhard and Wieschaus, 1980; Qian *et al.*, 1993). The third type of gene is the segment polarity genes whose mutations lead to a deletion in part of every segment and a replacement of the deleted part with a mirror image of the remaining structure (Nusslein-Volhard and Wieschaus, 1980; Hooper and Scott, 1989; Perrimon and Smouse, 1989). Correct spatial and temporal expression of the *Hom* genes is required for segment identity in the developing *Drosophila* embryo. The segmentation genes serve to initiate a transcriptionally active (by the pair-rule genes) or repressed (by the gap genes) state of the *Hom* genes (Scott and Carroll, 1987). Early in embryogenesis (about four hours), expression of the segmentation genes disappear (Akam, 1987), and other regulatory mechanisms are required to maintain the transcriptional program created by these genes.

The polycomb group proteins and trithorax group proteins maintain the transcriptional state of the *Hom* genes initiated by the segmentation genes. PcG proteins maintain repression of the *Hom* genes while trithorax group proteins maintain their activation. Mutations of PcG proteins lead to a derepression of *Hom* genes and segments that differentiate into structures characteristic of posterior segments (Struhl and Akam, 1985; Simon *et al.*, 1992; Chiang *et al.*, 1995). Ectopic expression or mutation of *Hom* genes in *Drosophila* changes the identity of the segment and leads to the formation of structures characteristic of a different segment (Kaufman *et al.*, 1990; Pattatucci and Kaufman, 1991; Pattatucci *et al.*, 1991). Polycomb and trithorax group proteins play a vital role in transcriptional regulation of the homeotic genes in *Drosophila* during embryonic development.

2. Regulation of Hox genes in mammals.

The *Hox* genes play a similar role in mammalian development. *Hox* genes are required for patterning of the body plan and development of the axial skeleton. Similar to *Drosophila*, the mammalian axial skeleton develops from metameric units called somites. During gastrulation, a subpopulation of the mesoderm that resides around the neural tube (paraxial mesoderm) is formed. Somites form from blocks of cells that separate from the paraxial mesoderm in a process called “initial segmentation.” The dorsal portion of the somite eventually becomes the dermomyotome. The ventral portion becomes the sclerotome, which contains stem cells that eventually give rise to the axial skeleton. In a process called “resegmentation,” the sclerotome cells segregate into rostral and caudal compartments. The rostral and caudal halves then fuse with their neighbor to form a vertebra (reviewed in (Yamaguchi, 1997; Saga and Takeda, 2001).

Hox genes regulate the patterning of the mammalian axial skeleton. While *Drosophila* have only two clusters of *Hox* genes (Antennapedia complex – ANT-C and bithorax complex – BX-C), mammals have thirty-nine *Hox* genes arranged in four clusters (*HoxA*, *HoxB*, *HoxC*, and *HoxD*) and thirteen paralogous groups (*Hox1-13*). The expression of *Hox* genes is described as “temporal and spatial colinearity.” This term refers to how the timing of expression during embryonic development correlates with the spatial location of the *Hox* genes within each cluster. The 3' genes (*HoxA1*, *HoxB1* etc...) are expressed the earliest and are detected at seven days post-conception (dpc) (Dolle *et al.*, 1989; Izpisua-Belmonte *et al.*, 1991). Mutations in 3' *Hox* genes exhibit phenotypes in the anterior region of the axial skeleton, while mutations in 5' *Hox* genes exhibit phenotypes in the posterior region (Condie and Capecchi, 1993; Davis and Capecchi,

1994). Due to the redundancy within paralogous Hox genes, mutation of any single *Hox* gene do not affect viability and result in relatively minor phenotypes. Mutations of the *Hox3* through *Hox11* genes cause defects in the axial skeleton (Chisaka and Capecchi, 1991; Le Mouellic *et al.*, 1992; Condie and Capecchi, 1993; Jeannotte *et al.*, 1993; Davis and Capecchi, 1994; Rancourt *et al.*, 1995; Suemori *et al.*, 1995; Chen and Capecchi, 1997; van den Akker *et al.*, 1999; van den Akker *et al.*, 2001; McIntyre *et al.*, 2007). Paralogous mutations have been constructed for many of the *Hox* gene groups. These mutations lead to synergistic and severe axial skeletal phenotypes. For example, mutations of the *Hox9* paralogous group have anterior transformations of the thoracic vertebrae, such that there are thirteen or fourteen ribbed vertebra attached to the sternum instead of the normal seven (McIntyre *et al.*, 2007). Mutation of an entire cluster has a less severe phenotype than paralogous mutations. The exception to this is *HoxB*, whose mutation results in a severe sternal phenotype (Medina-Martinez *et al.*, 2000). This is probably due to the fact that *HoxB* contains *Hox5-9*, all of which are important for the patterning of the sternum. The other *Hox* clusters have only two to four members of *Hox5-9*. It is clear from these studies that coordinated expression of the *Hox* genes is vital to proper development of the mammalian embryo. Thus, it is important to understand the mechanisms underlying the regulation of these genes.

Hox gene regulation in mammals is more complex than in *Drosophila*. Gap or pair-rule type regulation has not been identified in mammals. Instead, *Hox* regulation is initiated by a variety of transcription factors, signaling molecules, and polycomb group proteins. During development, FGF (Ciruna and Rossant, 2001; Dubrulle and Pourquie, 2004), WNT (Aulehla *et al.*, 2003; Forlani *et al.*, 2003) and retinoic acid (Boncinelli *et*

al., 1991; Krumlauf, 1994) gradients are early regulators of *Hox* genes. These molecules act by signaling to *Cdx* genes, which then directly stimulate the transcription of *Hox* genes in a dosage dependent manner (Subramanian *et al.*, 1995; Pownall *et al.*, 1996; Charite *et al.*, 1998; Isaacs *et al.*, 1998; Houle *et al.*, 2003; Gaunt *et al.*, 2004). Mutations of *Cdx* genes, hypomorphs of *Fgfr1*, and hypomorphs of *Wnt3* lead to a deregulation of *Hox* genes and axial skeletal transformations (Partanen *et al.*, 1998; Ikeya and Takada, 2001; van den Akker *et al.*, 2002). The mechanisms described above serve to initiate transcriptional activation of the *Hox* genes. Transcriptional repression of these genes early in embryonic development requires the actions of multiprotein polycomb repressive complexes.

3. Polycomb Repressive Complexes

Two core Polycomb repressive complexes (PRCs) have been purified from *Drosophila* and HeLa cells and were found to have many of the same homologous proteins. PRC2, which is required for the initiation of *Hox* gene repression in mammals, contains the proteins EZH2, Suz12, and EED (Cao *et al.*, 2002; Kuzmichev *et al.*, 2002; Muller *et al.*, 2002). This complex initiates a heritable, repressive state of *Hox* genes through modifications of the chromatin structure. EZH2, a core component of the PRC2 complex methylates histone H3 at lysine 27 (Cao *et al.*, 2002; Czermin *et al.*, 2002; Kuzmichev *et al.*, 2002; Muller *et al.*, 2002). In some cases, this methyl mark serves to recruit PRC1. However, it has also been shown that PRC1 is recruited in the absence of PRC2 (Schoeftner *et al.*, 2006). The PRC1 core complex contains the proteins Bmi1, Ring1a, Ring1b, HPH, HPH2, HPC2, HPC3, and Scmh1 (Saurin *et al.*, 2001; Levine *et al.*, 2002). Most importantly, HPC, a homologue of the *Drosophila* polycomb (Pc)

protein, directly binds to methylated histone H3 at lysine 27 through a highly conserved chromodomain (Cao *et al.*, 2002; Muller *et al.*, 2002). PRC2 initiates *Hox* gene repression, and PRC1 maintains the repression throughout development. Mice deficient for a component of the PRC2 complex die early in development by 7 dpc (Faust *et al.*, 1995; O'Carroll *et al.*, 2001; Pasini *et al.*, 2004). In contrast, most PRC1 mice exhibit no reduction in viability and display axial skeletal transformations (van der Lugt *et al.*, 1994; Takiyara *et al.*, 1997; del Mar Lorente *et al.*, 2000; Tokimasa *et al.*, 2001). The exception to this is *Ring1b* (*Ring2*) deficient mice. These mice arrest in gastrulation, and they are predicted to have a proliferation defect. An upregulation of the *Ink4a-Arf* locus is observed, and mice deficient for both *Ring1b* and *Ink4a-Arf* exhibit a partial rescue of the early embryonic lethality (Voncken *et al.*, 2003).

IV. Polycomb Group Proteins in Development and Disease.

Polycomb group proteins are required for correct development and patterning of the mammalian embryo. Mutation or misexpression of these genes causes a wide spectrum of defects. These include early embryonic lethality, axial skeletal transformations, and a variety of cancers. PcG mutants also exhibit impairment in hematopoiesis, cell cycle control, senescence, X-inactivation, stem cell maintenance, and differentiation. Described below are some of the key players of polycomb group complexes and polycomb group proteins that have been found to biochemically interact with E2F6.

1. PRC2: EZH2, EED, Suz12

The PRC2 complex plays a vital role in the initiation of a developmental program that is required for patterning of the mammalian embryo. The components of the PRC2 complex are required for early embryonic development, and mice deficient for these genes die by seven days post-conception (Faust *et al.*, 1995; O'Carroll *et al.*, 2001; Pasini *et al.*, 2004). EZH2 is the component of the PRC2 complex that methylates histone H3 at lysine 27. Loss of EZH2 results in a loss of this methylation mark in early zygotes (Erhardt *et al.*, 2003) and severely compromises the proliferation of embryonic stem (ES) cells. Attempts to derive ES cells from blastocysts generate non-ES-like cells that become apoptotic or necrotic (O'Carroll *et al.*, 2001). Alternatively, overexpression of EZH2 increases the proliferative capacity of primary B cells (Visser *et al.*, 2001) and bypasses cellular senescence in MEFs (Kamminga *et al.*, 2006). EZH2 is strongly expressed in highly proliferative, undifferentiated cells (Visser *et al.*, 2001). As cells

differentiate, expression of EZH2 is developmentally downregulated, and overexpression of EZH2 in undifferentiated cells prevents them from differentiating (Carette *et al.*, 2004). One hypothesis is that EZH2 plays a role in maintaining a cell's "stemness" during development. In support of this hypothesis, overexpression of EZH2 in hematopoietic stem cells prevents stem cell exhaustion when bone marrow is serially transplanted (Kamminga *et al.*, 2006). EZH2 is also found to be overexpressed in a variety of cancerous cells including lymphoma (Visser *et al.*, 2001), bladder cancer (Arisan *et al.*, 2005), breast carcinomas, prostate cancer (Varambally *et al.*, 2002; Kleer *et al.*, 2003), and bronchial squamous cell carcinomas (Breuer *et al.*, 2004). The precise mechanism of EZH2's involvement in cancer is unknown. An attractive model is that overexpression of EZH2 may be trapping the cell in a highly proliferative, stem cell-like state. The result is a block in appropriate differentiation and an induction of abnormal proliferation.

EED and Suz12 also play a role in differentiation and early embryonic development. Mice with a hypomorph for *Eed* exhibit posterior transformations along the AP axis (Schumacher *et al.*, 1996) and a deregulation of *Hox* genes (Wang *et al.*, 2002), consistent with a role for this protein in *Hox* gene regulation. *Eed* homozygous mutant mice display a defect in embryonic ectoderm growth, an absence in axial structures, and early embryonic lethality (Niswander *et al.*, 1988; Faust *et al.*, 1995). Unlike EZH2 mutant ES cells, ES cells from *Eed* or *Suz12* mutant blastocysts are viable, but they lack methylation of histone H3 at lysine 27 (Montgomery *et al.*, 2005; Boyer *et al.*, 2006; Pasini *et al.*, 2007). Suz12 plays an essential role in promoting differentiation. ES cells and embryo bodies deficient for this gene fail to differentiate properly (Pasini *et al.*, 2004; Pasini *et al.*, 2007). Additionally, a Suz12 point mutation that causes aberrant

mRNA splicing results in an increase in the number of multipotent hematopoietic progenitors and enhances hematopoietic stem cell activity (Majewski *et al.*, 2008). These results present a requirement for Suz12 and the PRC2 complex in differentiation of different populations of progenitor cells.

2. Bmi1

Bmi1 was first identified as an oncogene that cooperates with the E μ -myc transgene in B-cell lymphoma (Haupt *et al.*, 1991; van Lohuizen *et al.*, 1991). Bmi1 is homologous to the *Drosophila* gene posterior sex combs, (Adler *et al.*, 1991; Brunk *et al.*, 1991) and it has been found to be a part of the core PRC1 complex involved in maintenance of gene repression (Lewis, 1978; Paro, 1990; Zink *et al.*, 1991; Saurin *et al.*, 2001; Levine *et al.*, 2002). Bmi1 is highly expressed progenitor cell populations. Adult tissues have a low level of Bmi1 RNA with the exception of the thymus, heart, brain, and testes (van Lohuizen *et al.*, 1991). Mice deficient for this protein exhibit a variety of axial skeletal transformations, which is consistent with a role for Bmi1 in *Hox* gene repression. The transformations include (van der Lugt *et al.*, 1994; Courel *et al.*, 2008):

- (1) E – an extra piece of bone rostral to the first cervical vertebra, C1
- (2) C1-C2 conversion in which the second cervical vertebra is transformed to the first cervical vertebra and has an axis-like appearance
- (3) C7-T1 conversion evidenced by the presence of ribs on the seventh cervical vertebra, which then fuse with ribs at the first thoracic vertebra or connect directly to the sternum
- (4) T7-T8 conversion resulting in six vertebrosteral ribs instead of seven

(5) T13-L1 conversion in which there is an absence or incomplete development of ribs normally present on the thirteenth thoracic vertebra and

(6) L6-S1 conversion demonstrated by an association of iliac bones with the sixth lumbar vertebra instead of the first sacral vertebra.

In addition to axial skeletal transformations, *Bmi1*-mutant mice display a variety of other defects, indicating that *Bmi1* has roles beyond the maintenance of repression of the *Hox* genes.

Bmi1-mutant mice are born at expected frequency but are smaller in size and selectively cannibalized shortly after birth. Approximately 50% survive to adulthood (van der Lugt *et al.*, 1994; Courel *et al.*, 2008). In addition to axial skeletal transformations, these mice also display defects in hematopoiesis, the central nervous system, and the peripheral nervous system (van der Lugt *et al.*, 1994; Jacobs and van Lohuizen, 2002). In the hematopoietic system, there is a loss of mature T and B cells, hypocellularity of the bone marrow, decreased spleen size, and an involuted thymus (van der Lugt *et al.*, 1994; Lessard and Sauvageau, 2003). The neurological defects in the *Bmi1* mutant mice include an ataxic gait; seizures; hypocellularity of the molecular and granular layers of the cerebellum; and astrogliosis in the cortex and cerebellum of the brain (van der Lugt *et al.*, 1994; Molofsky *et al.*, 2003; Leung *et al.*, 2004; Zencak *et al.*, 2005). The neurological and hematopoietic defects can be partially attributed to a deficiency in the proliferation and self-renewal capacity of the stem cells in these compartments (Lessard and Sauvageau, 2003; Park *et al.*, 2003; Molofsky *et al.*, 2005).

The proliferation defects observed *in vivo* are consistent with a role for the PcG proteins in the control of the cell cycle. Indeed, mouse embryonic fibroblasts (MEFs)

deficient for *Bmi1* have impaired proliferation properties and undergo premature senescence (Core et al., 1997; Jacobs et al., 1999b; Courel et al., 2008). *Bmi1* is a repressor of the *Ink4a-Arf*, and the cell cycle defects in the *Bmi1*^{-/-} MEFs result from a derepression of this locus (Jacobs et al., 1999b). Mice mutant for *Bmi1* and *Ink4a-Arf* have a partial rescue of the neural and hematopoietic defects. *Bmi1*^{-/-};*Ink4a-Arf*^{-/-} mice exhibit a cerebellum that is comparable in size to wildtype mice, and the number of thymocytes and splenocytes is now 50-70% of wildtype levels. The proliferative and senescent defects of MEFs deficient for *Bmi1* are fully rescued in *Bmi1*^{-/-};*Ink4a-Arf*^{-/-} MEFs (Jacobs et al., 1999b). These studies clearly show that *Bmi1* plays a vital role in the INK4A-ARF tumor surveillance pathway. Therefore, it is not surprising that *Bmi1* is deregulated in a variety of cancers. These cancers include: high grade B-cell Non-Hodgkin lymphomas (Bea et al., 2001), breast carcinomas (Dimri et al., 2002), non-small cell lung cancers (Vonlanthen et al., 2001), medulloblastomas (Leung et al., 2004), and human colorectal cancers (Kim et al., 2004). Recent studies implicate *Bmi1* in the proliferation of bronchiolalveolar stem cells and a requirement for *Bmi1* in lung tumorigenesis (Dovey et al., in press). It is clear that *Bmi1* and INK4a-ARF are important for the maintenance and proliferation of different populations of stem cells, and deregulation of these genes can cause inappropriate proliferation and cancer.

3. Mel-18

Mel-18 is a PcG protein that is 70% identical to *Bmi1* (Tagawa et al., 1990; Goebel, 1991). Like the *Bmi1* mutant mice, *Mel-18* mutant mice are growth retarded and defective in hematopoiesis. These mice also die in a similar time frame as the *Bmi1* mutant mice (Akasaka et al., 1996). The hematopoietic defects of *Mel-18* mutant mice

include a defect in B-cell proliferation and maturation, impaired expansion of the most immature T progenitor cells, severe thymic atrophy, and an impairment in the self-renewal and proliferation of the hematopoietic stem cells (Akasaka *et al.*, 1996; Akasaka *et al.*, 1997; Miyazaki *et al.*, 2005). *Mel-18*^{-/-} mice also exhibit posterior axial skeletal transformations similar to those found in *Bmi1* deficient mice. These include E, C1-C2, C7-T1, T13-L1, and L6-S1. These mice also have a C2-C3 transformation marked by the lack of the odontoid process from the C2 vertebra and an S4-Ca1 transformation in which the fourth sacral vertebra looks like the first caudal vertebra (Akasaka *et al.*, 1996). It is apparent that *Bmi1* and *Mel-18* have overlapping functions *in vivo*. However, there are no indications that *Mel-18* is a regulator of the *Ink4a-Arf* locus or that it plays a role in the proliferation of neural or lung stem cells.

4. Ring1/Ring1a and Ring2/Ring1b

Ring1a and *Ring1b* were first characterized on the basis of their interaction with *M33* in a yeast two-hybrid screen (Schoorlemmer *et al.*, 1997). The two genes are both found to be in the PRC1 core complex, and they interact with many other PcG proteins (Satijn *et al.*, 1997; Schoorlemmer *et al.*, 1997; Hemenway *et al.*, 1998; Satijn and Otte, 1999; Levine *et al.*, 2002). *Ring1a* deficient mice are viable, but they display axial skeletal transformations (del Mar Lorente *et al.*, 2000). Interestingly, the skeletal transformations in *Ring1a* mutant mice are anterior, while all other mutant mice for PRC1 components have posterior transformations (van der Lugt *et al.*, 1994; Akasaka *et al.*, 1996; Takihara *et al.*, 1997). The exception to this is *M33* mutant mice, in which most of the axial skeletal transformations are posterior except for an anterior transformation of

C2-C1 (Core *et al.*, 1997; Katoh-Fukui *et al.*, 1998). The anterior axial skeletal transformations of *Ring1a* mutant mice include (del Mar Lorente *et al.*, 2000):

- (1) an abnormal C1 and C2
- (2) a C2-C1 transformation characterized by a broadening of the neural arch
- (3) a T3-T2 transformation in which the prominent spinous process normally found on T2 is now present on T3
- (4) a T8-T7 transformation marked by the presence of eight vertebrosteral ribs instead of seven and
- (5) a L1-T13 transformation evidenced by rudimentary ribs on L1.

Mice overexpressing *Ring1a* display many of the same transformations, including the C2-C1, the T8-T7, and the L1-T13. These mice also have a T10-T9 transformation (dorsal cartilage normally found on T10 is now present on T9) (del Mar Lorente *et al.*, 2000). Expression of *Ring1a* in the embryo is limited to the central nervous system at E8.5. At E13.5 it is found in the central and peripheral nervous system, and at E15.5 it is also found in the thymus and epithelial cell types. In the adult mouse, *Ring1a* is expressed in differentiated tissues (Schoorlemmer *et al.*, 1997). The skeletal transformations of *Ring1a* are consistent with its role as a member of PRC1.

In contrast to *Ring1a* mutant mice, *Ring1b* mutant embryos arrest early in embryogenesis during gastrulation (Voncken *et al.*, 2003). *Ring1b* expression is found early in the blastocyst and embryonic stem cells (Voncken *et al.*, 2003). A *Ring1b* hypomorph mutant mouse is viable, but it displays posterior axial skeletal transformations. These include (Suzuki *et al.*, 2002):

- (1) a supraoccipital bone-C1 transformation in which there are ectopic bones on the C1 vertebra
- (2) a C1-C2 transformation
- (3) a C2-C3 transformation in which C2 lacks the odontoid process
- (4) a C7-T1 transformation
- (5) a T1-T2 transformation in which the prominent spinous process of T2 is now present on T1
- (6) a T7-T8 transformation
- (7) a T13-L1 transformation
- (8) a L6-S1 transformation and
- (9) a S4-Ca1 transformation.

These skeletal transformations were accompanied by a deregulation of *Hox* genes (Suzuki *et al.*, 2002). The diversity in the phenotypes and viability of the *Ring1a* and *Ring1b* mutant mice suggests that these two highly related proteins have different functions *in vivo*.

Little was known about the mechanism of Ring1a and Ring1b until de Napoles *et al.* found that these proteins have catalytic E3 ubiquitin ligase activity towards histone H2A at lysine 119. Ring1b maintains global H2A ubiquitination in ES cells, and both Ring1a and Ring1b can ubiquitinate histone H2A on the inactive-X chromosome (de Napoles *et al.*, 2004; Cao *et al.*, 2005). The big question was whether the ubiquitin chromatin mark contributes to repression or whether it has another function. To answer this question, Cao *et al.* found evidence that this chromatin mark participated in repression by PRCs and that H2AK119 ubiquitination is downstream of histone H3 lysine

27 methylation (Cao *et al.*, 2005). Recent evidence suggests the ubiquitin mark of histone H3 interferes with the processivity of the RNA polymerase. This provides a direct mechanism by which histone H3 ubiquitination represses transcription by interfering with the RNA polymerase (Stock *et al.*, 2007).

5. RYBP

RYBP (Ring1 and YY1 Binding Protein) was cloned as an interactor with Ring1a. RYBP also interacts with many other PcG proteins and E2Fs 2, 3, and 6 (Garcia *et al.*, 1999; Schlisio *et al.*, 2002). Recent published and unpublished results provide insight into the mechanism in which RYBP may contribute to polycomb silencing and X-inactivation. RYBP contains an Np14 zinc finger (NZF) and binds to ubiquitinated histone H2A *in vivo* (Arrigoni *et al.*, 2006). Additionally, RYBP co-localizes with Ring1a at the inactive-X chromosome (Arrigoni *et al.*, 2006). Experiments from Professor Jackie Lees's laboratory identified Suv4-20 in a yeast two-hybrid assay as a possible interactor of RYBP. This protein, similar to EZH, contains a SET domain and can tri-methylate histone H4 at lysine 20 (Schotta *et al.*, 2004). This methyl mark, which is downstream of the methylation of histone H3 lysine 27, is found at pericentric heterochromatin and the inactive-X chromosome (Plath *et al.*, 2003; Silva *et al.*, 2003; Schotta *et al.*, 2004). One possible mechanism is a sequential recruitment of histone modifications that contribute to a highly condensed state of chromatin. RYBP may serve as a bridging protein between the complex that ubiquitinates histone H2A and the complex that methylates histone H4 at lysine 20. The interaction between RYBP and Suv4-20, however, has not been confirmed, and this mechanism is highly speculative.

Rybp mutant embryos die early during embryonic development at E5.5, indicating that RYBP is important during postimplantation. *Rybp*^{+/-} and chimeric mice have many defects of the central nervous system and in ocular development. These include exencephaly due to defective neural tube closure, chaotic forebrain outgrowth, retinal coloboma, ventral rotation of the lens, and an abnormal separation of the lens vesicle from the surface of the ectoderm. There is no indication that *Rybp*^{+/-} mice display any axial skeletal transformations or deregulation of *Hox* genes. Clearly, *Rybp* is essential for proper embryonic and central nervous system development, and it will be important to study the exact mechanism and the significance of RYBP's ubiquitin binding activity.

6. Synergy between PcG proteins

Compound mutants of PRC1 components have been generated in mice and flies. Despite the fact that these proteins participate in the same complex, the result is often an exacerbation of the skeletal and developmental defects (Bel *et al.*, 1998; Akasaka *et al.*, 2001; Kwon *et al.*, 2003; Courel *et al.*, 2008). This result emphasizes the partial functional redundancy of some PRC1 components. The synergy between PRC1 components is best studied in *Bmi1* mutant mice. These mice exhibit posterior axial skeletal transformations as well as severe developmental defects. The posterior axial skeletal transformations are due to deregulation of *Hox* genes, and the developmental defects are due to a deregulation of the *Ink4a-Arf* locus and impaired stem cell proliferation and maintenance.

Compound mutants of *Bmi1*, a core component of PRC1, and other PRC1 proteins show a clear synergy in *Hox* gene regulation. Mice doubly deficient for *Bmi1* and *Mel18* or *M33* exhibit exacerbated defects in axial skeletal development. Significantly, the

severity of these defects increases upon a decrease in the PcG gene dosage. These compound mutant mice also show an increased anterior shift in the boundaries of some *Hox* genes in the embryos (Bel *et al.*, 1998; Akasaka *et al.*, 2001). Mice doubly deficient for *Bmi1* and *E2f6* have also been generated. There is an increased penetrance of some of the axial skeletal transformations, and this is accompanied by a further derepression of *Hox* genes. E2F6 was also found to occupy a subset of *Hox* gene promoters that *Bmi1* occupies (Courel *et al.*, 2008). *Bmi1* loss in combination with mutation of *E2f6*, *Mel-18*, or *M33* causes further deregulation of *Hox* genes and exacerbated axial skeletal defects. These exemplified genetic interactions underscore the documented biochemical interactions for these proteins and provide a biologically relevant role for this interaction.

E2F6, M33, and Mel-18 synergize with *Bmi1* in axial skeletal development, but there is no conclusive evidence to suggest that these proteins cooperate with *Bmi1* in the regulation of *Ink4a-Arf*. Mice doubly deficient for *Bmi1* and *Mel18* or *M33* die during embryogenesis (Bel *et al.*, 1998; Akasaka *et al.*, 2001). The *Bmi1*;*M33* compound mutant mice exhibit increased apoptosis in the embryo, suggesting that there may be enhanced deregulation of the *Ink4a-Arf* locus. These mice did have a ten-fold increase in *Arf* levels compared to wildtype littermates, but the levels in *Bmi1* mutant embryos were not reported in this analysis. It is inconclusive whether the levels of *Arf* are further increased in the compound mutant embryos when compared to the *Bmi1* mutant embryos or whether the level of derepression seen was solely due to loss of *Bmi1*. No analysis was done of cells from these mice, and it is unknown whether loss of *Mel-18* in *Bmi1*-mutant mice enhances the proliferation defect of primary cells (Akasaka *et al.*, 2001). In the case of the *Bmi1*;*M33* compound mutant mice, no analysis was done of the *Ink4a-Arf* locus,

apoptosis, or proliferation, and there was no suggestion as to why these mice died significantly earlier than the *Bmi1* or *M33* single mutants (Bel *et al.*, 1998).

In contrast to *M33* and *Mel18*, loss of *E2f6* in *Bmi1* mutant mice does not alter the lifespan of these mice. In this case, an interaction between E2F6 and Bmi1 in the regulation of *Ink4a-Arf* was well studied. Compound mutants, as well as intermediate genotypes, were analyzed for exacerbated hematopoietic and neural phenotypes. There was no significant difference between the *Bmi1*-mutant and *Bmi1;E2f6* compound mutant mice. MEFs from these mice did not have enhanced proliferative defects or enhanced deregulation of Bmi1 or E2F6 target genes. The exception to this is a subset of the *Hox* genes, in which the penetrance was increased in *Bmi1^{-/-};E2f6^{-/-}* mice compared to *Bmi1^{-/-}* mice. Additionally, Bmi1, but not E2F6, was found to occupy the promoter of *Arf* in wildtype embryonic stem cells. These data clearly indicate that E2F6 does not play a role in the regulation of the *Ink4a-Arf* locus (Courel *et al.*, 2008).

These studies of the genetic interactions between PRC1 components consistently show that loss of one or two PRC1 components results in a deregulation of some *Hox* genes. This result underscores the complexity of *Hox* gene regulation and suggests that there may be PRCs of different compositions that regulate different *Hox* genes. Many of these proteins are involved in modification of histones and chromatin compaction. It is likely that these chromatin marks play an important role in the regulation of *Hox* and other genes. It will be important to do a more detailed analysis of these chromatin marks and the complexes that initiate and maintain them.

V. Reactive Gliosis

The E2F proteins are classically known for their role in regulating the cell cycle, however, these proteins play other roles in development and disease. In this thesis, I have described a novel requirement for E2F6 in reactive gliosis. Reactive gliosis forms in response to brain injury and central nervous system pathologies including Parkinson's disease, Alzheimer's, stroke, amyotrophic lateral sclerosis, and pathological pain (Abraham, 2001; Teismann *et al.*, 2003; Barbeito *et al.*, 2004; Swanson *et al.*, 2004; Teismann and Schulz, 2004; Wieseler-Frank *et al.*, 2004).

Reactive gliosis refers to cellular changes that astrocytes undergo following brain trauma. Astrocytes are the most abundant cell population in the brain, and they have many supportive functions for neurons. Activation of astrocytes is characterized by an increase in the size of the cell body and processes and an increase in the levels of an intermediate filament protein called GFAP (glial fibrillary acidic protein). The most important questions are what role gliosis plays in brain trauma and neurodegeneration and whether this process is protective or harmful to the recovery process. There is evidence to suggest that gliosis is both neuroprotective and harmful. Astrocytes have neuroprotective properties in the absence of trauma, and these properties are especially important upon neuronal injury. After acute neuronal damage to the central nervous system, there is a release of glutamate from neurons (Swanson *et al.*, 2004). Glutamate and potassium uptake by astrocytes prevents toxic elevations of these molecules in the brain and extracellular space (Aschner and Kimelberg, 1991; Anderson and Swanson, 2000). Neurodegeneration is also associated with a high level of oxidative stress (Sano *et al.*, 1997; Marcus *et al.*, 1998). Astrocytes contain the highest concentration of

antioxidants and protect neurons from oxidative stress (Dringen, 2000; Dringen and Gutterer, 2002). During both normal brain function and trauma, astrocytes play a vital role in scavenging potentially toxic agents from the extracellular matrix.

Studies from *in vivo* mouse models that disrupt the intermediate filament proteins reveal that gliosis may inhibit recovery after brain trauma. *GFAP*^{-/-} mice still exhibit a normal gliotic response to stab wound or scrapie infection (Gomi *et al.*, 1995; Pekny *et al.*, 1995). This is most likely due to the redundancy with another intermediate filament, vimentin (VIM). Mice deficient for both *GFAP* and *Vim* exhibit a defect in post-traumatic glial scarring and a slower healing process (Pekny *et al.*, 1999). These mice also have remarkable synaptic regeneration relative to wildtype controls (Ishikawa *et al.*, 1997; Koyama *et al.*, 1999), and astrocytes isolated from these mice are a better substrate for outgrowth of neurites *in vitro* (Menet *et al.*, 2000). Cell proliferation, cell survival, and neurogenesis in the hippocampus of old *GFAP*^{-/-}; *Vim*^{-/-} mice increased 30% compared to wildtype controls (Larsson *et al.*, 2004). This suggests that age-related gliosis may restrict neurogenesis in old individuals and that blocking gliosis in this setting has many potential benefits.

Gliosis may exacerbate the pathologies of individuals with neurodegenerative disease and may impede neural repair. There is evidence to suggest that inhibiting gliosis in mice with neurodegeneration disorders or ischemia improves the pathology of these mice. Anti-inflammatory drugs such as ibuprofen have been shown to suppress the gliotic response *in vivo* in a mouse model for Alzheimer's disease and ischemia. Transgenic mice overexpressing the amyloid precursor protein undergo many of the same pathological changes as individuals with Alzheimer's disease (Sturchler-Pierrat *et al.*,

1997). When these mice are administered ibuprofen, the amyloid plaque formation is greatly reduced, and the gliotic response is blocked (Lim *et al.*, 2000; Jantzen *et al.*, 2002; Yan *et al.*, 2003). Similarly, dispensing ibuprofen to mice or rats subjected to ischemia reduces neuronal injury and improves cerebral blood flow and neurological outcome (Kuhn *et al.*, 1986; Cole *et al.*, 1993; Patel *et al.*, 1993; Antezana *et al.*, 2003). These studies suggest that blocking the gliotic response in individuals with neurodegeneration may be a successful therapeutic option.

It is clear that gliosis is involved in neurodegenerative disorders and brain trauma. However, the molecular mechanisms underlying this process are poorly understood. There is recent evidence to suggest that activation of the epidermal growth factor receptor (EGFR) signaling pathway triggers astrocyte activation and reactive gliosis. In the developing central nervous system, the EGFR regulates astrocyte migration and differentiation of neural precursor cells into astrocytes (Miettinen *et al.*, 1995; Sibilio and Wagner, 1995; Kornblum *et al.*, 1998). This pathway is reactivated during neurodegeneration, ischemia, and tumorigenesis (Birecree *et al.*, 1988; Planas *et al.*, 1998; Jin *et al.*, 2002; Hayashi *et al.*, 2004). EGFR regulates genes related to reactive astrocytes and neural disorders. These genes include those involved in extracellular matrix organization, cell migration, and cytokine production (Liu and Neufeld, 2004). In response to EGFR, astrocytes produce transforming growth factor β , brain-derived neurotrophic factor, fibroblast growth factor 2, and nerve growth factor β , which may all be beneficial to the survival of neurons (Liu *et al.*, 2006). However, activation of EGFR generates chondroitin sulfate proteoglycans and laminin, both of which interfere with axon regeneration after neural injury (McKerracher *et al.*, 1996; Anderson *et al.*, 1998;

Koprivica *et al.*, 2005). One theory is that neural injury induced by neurodegenerative diseases, tumorigenesis, and ischemia initiates inappropriate activation of a EFR-driven developmental program (Liu *et al.*, 2006). This model explains the duality of gliosis and suggests that this early developmental program is harmful to neurons in adult individuals.

Most of the research done on gliosis concludes that this process does more harm than good. For this reason, it is not surprising that many therapeutic options for neurodegenerative diseases and ischemia involve inhibition of gliosis. A better understanding of the molecular and cellular mechanisms that contribute to gliosis will lead to more effective and targeted therapies for these diseases.

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Chapter 2

E2f6 and Bmi1 cooperate in axial skeletal development

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ABSTRACT

Bmi1 is a Polycomb Group protein that functions as a component of Polycomb Repressive Complex 1 (PRC1) to control axial skeleton development through *Hox* gene repression. Bmi1 also represses transcription of the *Ink4a-Arf* locus and is consequently required to maintain the proliferative and self-renewal properties of hematopoietic and neural stem cells. Previously, one E2F family member, E2F6, has been shown to interact with Bmi1 and other known PRC1 components. However, the biological relevance of this interaction is unknown. In this study, we use mouse models to investigate the interplay between E2F6 and Bmi1. This analysis shows that E2f6 and Bmi1 cooperate in the regulation of *Hox* genes, and consequently axial skeleton development, but not in the repression of the *Ink4a-Arf* locus. These findings underscore the significance of the E2F6-Bmi1 interaction *in vivo* and suggest that the *Hox* and *Ink4a-Arf* loci are regulated by somewhat different mechanisms.

INTRODUCTION

The E2F transcription factors are a family of key regulators of cell proliferation and differentiation (Dyson, 1998; Nevins, 1998; Trimarchi and Lees, 2002). They act by controlling the transcription of genes whose expression is essential for cell cycle progression and DNA synthesis. In mammals, eight *E2f* genes (*E2f1-8*) have been identified. *E2f1-5* encode proteins that function as transcriptional activators or repressors by virtue of their association with the family of pocket proteins, pRB, p107, and p130. *E2f6-8* are more distantly related members of the E2F family. In particular, they lack the domain required for pocket protein-binding and are therefore not susceptible to pocket protein regulation (Cartwright *et al.*, 1998; de Bruin *et al.*, 2003; Gaubatz *et al.*, 1998; Logan *et al.*, 2004; Maiti *et al.*, 2005; Morkel *et al.*, 1997; Trimarchi *et al.*, 1998; Trimarchi *et al.*, 2001). E2F6 binds DNA as a heterodimer with DP, in a similar manner to E2F1-5, but due to the absence of a transactivation domain it does not activate transcription (Cartwright *et al.*, 1998; Gaubatz *et al.*, 1998; Morkel *et al.*, 1997; Trimarchi *et al.*, 1998;). Early studies showed that over-expressed E2F6 can repress classic E2F-responsive genes, at least in part, by binding to E2F-responsive promoters and blocking access to other activating E2Fs (Gaubatz *et al.*, 1998; Morkel *et al.*, 1997; Trimarchi *et al.*, 1998). Subsequently, E2F6 was shown to exist in complexes that contain both chromatin remodeling enzymes and members of the mammalian Polycomb Group (PcG), including Bmi1, Ring1, and RYBP (Attwooll *et al.*, 2004; Ogawa *et al.*, 2002; Trimarchi *et al.*, 2001). The PcG proteins, first identified in *Drosophila*, form large multimeric complexes that are responsible for the repression of the *Hox* genes, which determine the patterning of the developing embryo (Kennison 1995; Simon, 1995). The

documented interaction between E2F6 and PcG proteins suggested that E2F6 might actively enforce repression of E2F target genes *in vivo* through recruitment of the PcG complex(es) and might also participate in the regulation of known PcG-responsive targets.

In mammals, there are at least two distinct PcG complexes, called Polycomb Repressive Complexes (Otte and Kwaks, 2003). The Eed-containing PcG complex, PRC2, initiates polycomb-mediated repression, whereas the Bmi-1-containing PcG complex, PRC1, maintains the repression at later stages of development. Loss of proteins of the PcG-PRC2 in mice generally results in early embryonic lethality (Faust *et al.*, 1995; O'Carroll *et al.*, 2001) whereas PcG-PRC1 mutant mice typically survive until birth and display homeotic transformations of the axial skeleton (Akasaka *et al.*, 1996; Akasaka *et al.*, 1997; del Mar Lorente *et al.*, 2000; van der Lugt *et al.*, 1994). Notably, E2F6 has been shown to interact with known components of both PcG-PRC1 and PcG-PRC2 (Attwooll *et al.*, 2004; Ogawa *et al.*, 2002; Trimarchi *et al.*, 2001). Based on our previous association data (Trimarchi *et al.*, 2001), we have focused our attention on understanding the interplay between E2F6 and Bmi1.

Bmi1-deficient mice display defects in axial skeletal patterning, hematopoiesis, the central nervous system, and the peripheral nervous system (Jacobs and van Lohuizen, 2002; van der Lugt *et al.*, 1994). In the hematopoietic system, the stem cell defect results in a loss of mature T and B cells, hypocellularity of the bone marrow, decreased spleen size, and an involuted thymus (Lessard and Sauvageau, 2003; van der Lugt *et al.*, 1994). The neurological defects in the *Bmi1* mutant mice include an ataxic gait, seizures, hypocellularity of the molecular and granular layers of the cerebellum, and astrogliosis in

the cortex and cerebellum of the brain (Leung *et al.*, 2004; Molofsky *et al.*, 2003; van der Lugt *et al.*, 1994; Zencak *et al.*, 2005). The neurological and hematopoietic defects can be partially attributed to a deficiency in the proliferation and self-renewal capacity of the stem cells in these compartments (Lessard and Sauvageau, 2003; Molofsky *et al.*, 2005; Park *et al.*, 2003; Zencak *et al.*, 2005).

The proliferation defects observed *in vivo* are consistent with a role for the PcG proteins in the control of the cell cycle. Indeed, mouse embryonic fibroblasts (MEFs) deficient for *Bmi1*, *Mel-18* or *M33* have impaired proliferation properties and undergo premature senescence (Core *et al.*, 1997; Jacobs *et al.*, 1999). The cell cycle defects in the *Bmi1*^{-/-} MEFs result from the derepression of the *Ink4a-Arf* locus (Jacobs *et al.*, 1999). This locus encodes two proteins, p16^{INK4a} and p19^{ARF}, which regulate cellular proliferation and apoptosis. Only a few PcG proteins have been reported to bind DNA directly including Mel-18 and YY1 (Brown *et al.*, 1998; Kanno *et al.*, 1995). These proteins cannot account for all the Polycomb DNA binding activity suggesting that other DNA binding factors may facilitate the recruitment of PcG complexes to specific promoters. Notably, several E2F family members have been shown to play a direct role in the transcriptional regulation of p19^{ARF} (Aslanian *et al.*, 2004). Because E2F6 associates with PcG proteins and is able to directly bind DNA in a sequence-specific manner, we hypothesized that E2F6 might act to recruit PcG complexes to target promoters including *Arf*. To investigate the role of E2F6 in development and cell cycle control, we and others have generated *E2f6*-deficient mice (this study; Storre *et al.* 2002). Consistent with our prior observation that E2F6 interacts with Bmi1 and other PRC1 components (Trimarchi *et al.*, 2001), *E2f6*^{-/-} mice display subtle axial skeletal transformations. It is well

established that the combined mutation of two PcG-PRC1 components in flies or mice yields synergistic phenotypic effects (Adler *et al.*, 1991; Akasaka *et al.*, 2001; Bel *et al.*, 1998; Kwon *et al.*, 2003). Thus, to test for a possible genetic interaction between E2F6 and Bmi1, we have generated mice and MEFs that are deficient for both *E2f6* and *Bmi1* genes. Our data show that *E2f6*-deficiency increases the severity of the axial skeletal defect in *Bmi1* mutant mice but does not modulate the other *Bmi1* mutant phenotypes.

RESULTS

***E2f6* mutant mice are viable but display axial skeletal defects.**

In order to study the role of E2F6 *in vivo*, we generated an *E2f6* mutant mouse strain in which we have deleted a large proportion of the *E2f6* coding sequences including the exons that encode the DNA binding, leucine zipper and marked box domains (Figure 1A). Western blotting of *E2f6*^{-/-} MEFs confirms loss of the E2F6 protein (Figure 1B). Consistent with previous studies (Storre *et al.*, 2002) we find that *E2f6*-deficient mice are born at the expected frequency (120% of expected, n=233) and display no gross morphological defects. Previous studies have not assessed the lifespan in littermates. We generated a cohort of aging animals and found that there was no difference in the percentage of *E2f6*^{+/+}, *E2f6*^{+/-} versus *E2f6*^{-/-} mice that were alive after 530 days (70%, 68% and 75%, respectively; Figure 1C) or in the eventual cause of death of these animals. Thus, we conclude that *E2f6*-loss has no detectable effect on murine viability.

Figure 1

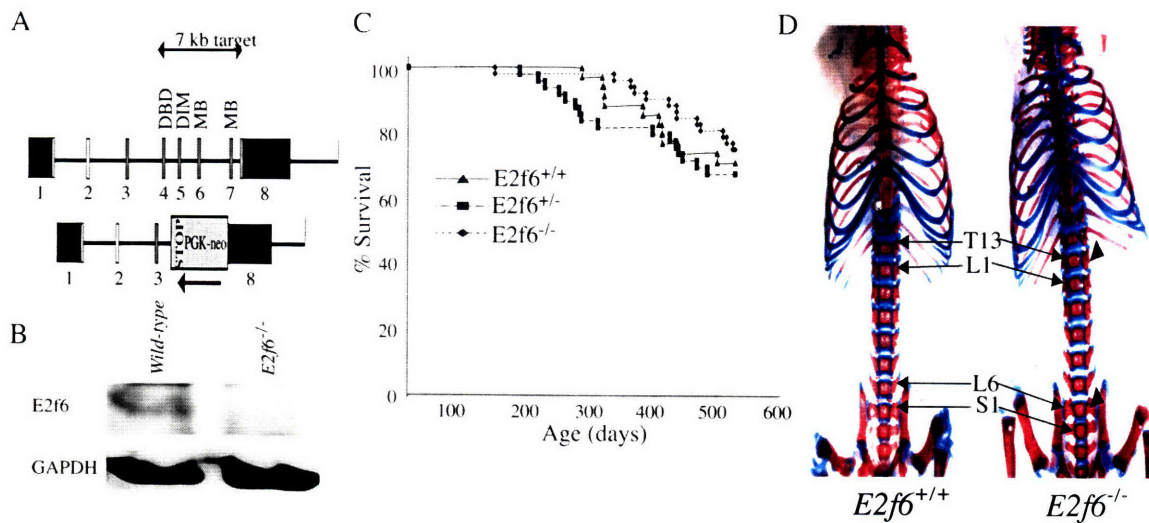


Figure 1: *E2f6* mutant mice are fully viable and display axial skeletal transformations.

(A) Generation of the *E2f6*^{-/-} mice. The *E2f6* genomic locus comprises 8 exons that include noncoding sequences (black boxes) coding sequences (gray boxes) and an alternatively spliced exon 2 (white box). DBD, DNA binding domain; DIM, dimerization domain; MB, marked box domain. The *E2f6* mutant allele was generated by replacing coding sequences of exons 4 through 8 with a PGK-neo cassette that includes a STOP codon at the beginning. PGK-neo, neomycin resistance gene under the regulation of the PGK promoter for positive selection. Disruption of the *E2f6* locus was confirmed by (B) Western of lysate from Wildtype and *E2f6*^{-/-} MEFs. (C) Survival curve of *E2f6* wildtype, heterozygote and mutant mice followed for over 500 days. (D) Ventral view of axial skeletons of newborn *E2f6*^{+/+} and *E2f6*^{-/-} mice stained with alcian blue (cartilage) and alizarin red (bone). *E2f6*^{-/-} mice display two axial skeletal transformations, the thirteenth thoracic vertebra (T13) is transformed into the first lumbar vertebra (L1) as shown by the degeneration of the thirteenth ribs and the sixth lumbar vertebra (L6) is transformed into the first sacral vertebra (S1) as evidenced by the formation of the sacral-iliac joints.

We have previously shown that E2F6 associates with Bmi1 and other components of the PcG-PRC1 complex *in vivo*. Because loss of Bmi1 in mice results in posterior transformations along the entire axial skeleton (van der Lugt *et al.*, 1994), we analyzed the skeletons of *E2f6*^{+/+}, *E2f6*^{+/-} and *E2f6*^{-/-} mice at postnatal day 3 (P3) by staining with Alcian blue and Alizarin red, which stain the cartilage in blue and the bone in red (Figure 1D). Similar to *Bmi1* and other PcG knockout mice (Akasaka *et al.*, 1996; van der Lugt *et al.*, 1994), *E2f6* mutant mice displayed posterior transformations. First, the thoracic vertebra T13 is transformed into a lumbar vertebra L1 as evidenced by the lack of ribs in 0% of *E2f6*^{+/+}, 9% of *E2f6*^{+/-}, and 67% of *E2f6*^{-/-} animals. Second, the lumbar vertebra L6 is transformed into the sacral vertebra S1 as shown by its association with the iliac bones in 0% of *E2f6*^{+/+}, 35% of *E2f6*^{+/-}, and 80% of *E2f6*^{-/-} mice (Figure 1D). These observations are consistent with those of Storre *et al.* (2002) who previously reported T13 to L1 and L6 to S1 transformations in a distinct *E2f6* mutant mouse model. Together, our data suggest that *E2f6* mutation results in dosage-dependent posterior transformations of the axial skeleton that are reminiscent of axial skeletal transformations seen in *Bmi1* and other PcG-PRC1 knockout mice.

Viability of *E2f6*^{-/-};*Bmi1*^{-/-} mice.

Compound mutants of PcG-PRC1 proteins have been generated in flies and mice resulting in dramatic synergistic effects (Adler *et al.*, 1991; Akasaka *et al.*, 2001; Bel *et al.*, 1998; Kwon *et al.*, 2003). Thus, to determine the biological relevance of the interaction between E2F6 and Bmi1, we have generated and analyzed *E2f6*^{-/-};*Bmi1*^{-/-} mice. *E2f6* deletion alone does not affect the viability and survival of mice. In contrast,

while *Bmi1*^{-/-} mice are born at the expected frequency, they are selectively cannibalized by their mothers shortly after birth and only approximately 50% survive into adulthood (van der Lugt *et al.*, 1994). These animals are significantly smaller than their wildtype littermates and display poor health that results in early lethality (3 to 20 weeks). In good agreement with this prior analysis, examination of the progeny from *E2f6*^{+/-};*Bmi1*^{+/-} intercrosses showed that *Bmi1*^{-/-} single mutant mice were underrepresented at 3 weeks of age (51% of expected: Table 1) but were present at near expected frequencies at embryonic day 18.5 (122% of expected: Table 2). In contrast, *E2f6*^{-/-};*Bmi1*^{-/-} mice arising

Table 1: Viability of *E2f6*;*Bmi1* compound mutant mice at three weeks of age.

n=250	<i>E2f6</i> ^{+/+} <i>Bmi1</i> ^{+/+}	<i>E2f6</i> ^{+/+} <i>Bmi1</i> ^{-/-}	<i>E2f6</i> ^{+/-} <i>Bmi1</i> ^{+/-}	<i>E2f6</i> ^{-/-} <i>Bmi1</i> ^{-/-}
Expected	15.63	15.63	31.25	15.63
Observed	16	8	24	6
% of expected	102	51	77	38

*Mice generated from *E2f6*^{+/-};*Bmi1*^{+/-} intercrosses and genotyped at three weeks of age.

Table 2: Viability of *E2f6*;*Bmi1* compound mutant mice at E18.5.

n=105	<i>E2f6</i> ^{+/+} <i>Bmi1</i> ^{+/+}	<i>E2f6</i> ^{+/+} <i>Bmi1</i> ^{-/-}	<i>E2f6</i> ^{+/-} <i>Bmi1</i> ^{+/-}	<i>E2f6</i> ^{-/-} <i>Bmi1</i> ^{-/-}
Expected	6.56	6.65	13.13	6.56
Observed	5	8	7	3
% of expected	76	122	53	46

* Mice generated from *E2f6*^{+/-};*Bmi1*^{+/-} intercrosses and genotyped at embryonic day 18.5.

from *E2f6*^{+/-};*Bmi1*^{+/-} intercrosses seemed to be underrepresented at both three weeks (38% of expected; Table 1) and also at E18.5 (46% of expected; Table 2). To better assess the relative viability of *E2f6*^{-/-};*Bmi1*^{-/-} versus *Bmi1*^{-/-} embryos, we conducted *E2f6*^{-/-}

;Bmi1^{+/-} intercrosses to generate the test genotypes at a much higher frequency. Analysis of the resulting progeny showed that there was no significant difference in the viability of *E2f6^{-/-};Bmi1^{-/-}* versus *Bmi1^{-/-}* embryos at E18.5. (Table 3; p=0.87). Therefore, loss of E2F6 has no effect on the viability of *Bmi1* mutant mice. In addition, the *E2f6^{-/-};Bmi1^{-/-}* mice showed a similar degree of growth retardation as their *Bmi1^{-/-}* littermates and these two genotypes both developed severe anemia and had to be sacrificed in a similar time window (data not shown).

Table 3: Viability of *E2f6:Bmi1* compound mutant mice at E18.5.

n=27	<i>E2f6^{-/-} Bmi1^{+/+}</i>	<i>E2f6^{-/-} Bmi1^{+/-}</i>	<i>E2f6^{-/-} Bmi1^{-/-}</i>
Expected	6.75	13.6	6.75
Observed	6	13	8
% of expected	89	96	118

* Mice generated from *E2f6^{-/-};Bmi1^{+/-}* intercrosses and genotyped at embryonic day 18.5.

E2F6 does not cooperate with *Bmi1* in the regulation of the INK4a-ARF locus.

To determine whether E2F6 and *Bmi1* play cooperating roles, we conducted a careful analysis of the cells and tissues that are known to be affected by *Bmi1*-loss. The lethal anemia of *Bmi1^{-/-}* mice results from a progressive decrease in the number of hematopoietic cells (van der Lugt *et al.*, 1994). Thus, we first compared the levels of various hematopoietic lineages in wildtype, *E2f6^{-/-}*, *Bmi1^{-/-}* and *E2f6^{-/-};Bmi1^{-/-}* mice at eight weeks of age. The mutation of *E2f6* alone had no detectable effect on the levels of hematopoietic cells or the distribution of the various white blood cell lineages (Figure 2).

In contrast, the *Bmi1*^{-/-} and *E2f6*^{-/-};*Bmi1*^{-/-} mice both showed a profound hematopoietic defect. First, we observed a significant reduction in the level of hematopoietic cells in the bone marrow, spleen, and thymus (Figure 2A). Second, there was a clear shift in the distribution of immature versus mature cells in various lineages. For example, in the thymus, there was a significant depletion of double positive (CD4+/CD8+) thymocytes, whereas immature (CD4-/IL2-R+, CD8-/IL2-R+, and CD4-/CD8-) and mature (CD4+/CD8- and CD4-/CD8+) cells are still present (Figure 2B). Moreover, in the bone marrow there was a significant shift in the distribution of myeloid (GR+/Mac-1+) versus B lymphoid cells in both the *Bmi1*^{-/-} and *E2f6*^{-/-};*Bmi1*^{-/-} mice. In each case, this leads to a higher percentage of myeloid cells, although the absolute number of myeloid cells is still reduced relative to wildtype (Figure 2C). Within the B cell population, we also observed a greater deletion of the immature B cells (B220+/HSA+; seven fold decrease in the percentage of cells) than the mature B cells (B220+/IgM+; three fold decrease in the percentage of cells) when compared with wildtype (Figure 2C). Similar results were seen in the spleen (data not shown). These changes are all consistent with the known defect in the maintenance and self-renewal capacity of the *Bmi1*^{-/-} hematopoietic stem cells . Importantly, there was no significant difference in the degree of these defects in the *E2f6*^{-/-};*Bmi1*^{-/-} versus the *Bmi1*^{-/-} mice, indicating that E2F6-loss does not modulate the effect of *Bmi1*-deficiency on the hematopoietic compartment.

Bmi1 deficient mice exhibit hypocellularity of various layers of the cerebellum and develop ataxia at the age of 2-4 weeks (Leung *et al.*, 2004; van der Lugt *et al.*, 1994). Thus, we tested whether the loss of E2F6 modulates these neuronal defects. First, we screened animals from *E2f6*^{+/-};*Bmi1*^{+/-} intercrosses that survived to 2 months of age for

Figure 2

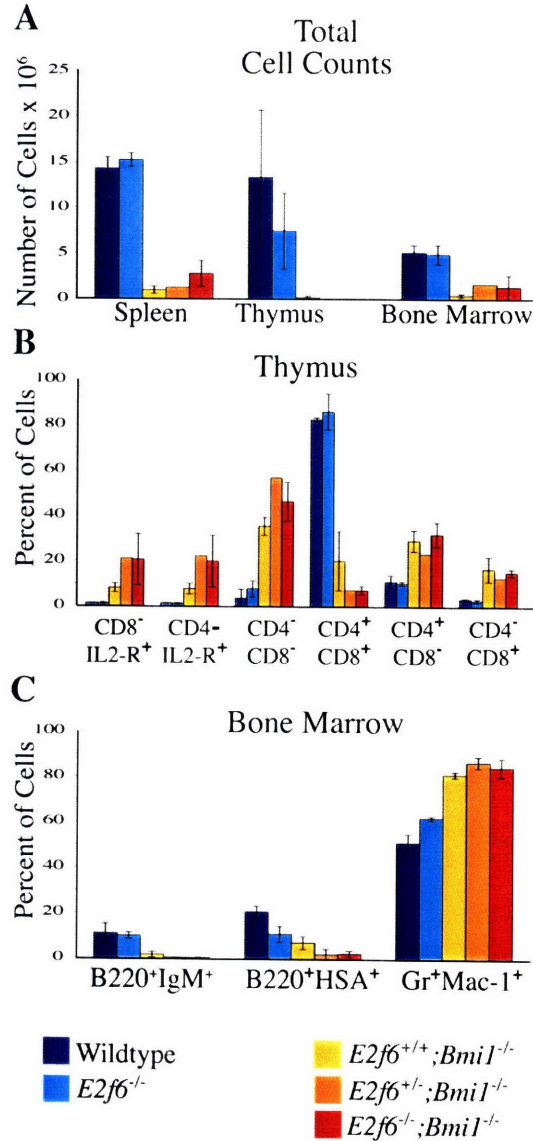


Figure 2: E2F6-loss does not modulate the hematopoietic defect within *Bmi1* mutant mice.

(A) Cell counts of single cell preparations made from the bone marrow, spleen, and thymus of eight week old mice. *E2f6*^{+/+};*Bmi1*^{+/+} n=2; *E2f6*^{-/-};*Bmi1*^{+/+} n=2; *E2f6*^{+/+};*Bmi1*^{-/-} n=3; *E2f6*^{+/-};*Bmi1*^{-/-} n=1; *E2f6*^{-/-};*Bmi1*^{-/-} n=3. FACS analysis of single cell preparations made from the thymus (B) and bone marrow (C) and immunostained with the indicated antibodies. Results are presented as percentage of cells. *E2f6*^{+/+};*Bmi1*^{+/+} n=2; *E2f6*^{-/-};*Bmi1*^{+/+} n=2 for part B, n=3 for part C; *E2f6*^{+/+};*Bmi1*^{-/-} n=3; *E2f6*^{+/-};*Bmi1*^{-/-} n=1 for part B, n=2 for part C; *E2f6*^{-/-};*Bmi1*^{-/-} n=3.

ataxia (data not shown). This defect was completely absent in animals that were either wildtype or *Bmi1*^{+/+}, irrespective of their *E2f6* status. In contrast, we observed ataxia in a similarly high proportion of the *Bmi1*^{-/-} (60%; n=4), *E2f6*^{+/+};*Bmi1*^{-/-} (65%; n=13) and *E2f6*^{-/-};*Bmi1*^{-/-} (70%; n=7). We did observe a general correlation between the degree of growth retardation and the likelihood that an animal would develop ataxia. It is unclear whether these defects are causally linked or whether they simply reflect some variation in the penetrance of *Bmi1* mutation in the mixed (C57/BL6 x 129S/v) genetic background of our animals. However, our data clearly show that *E2f6* status did not alter either the penetrance or the time of onset of the ataxia. To complement this analysis, we also performed a histological analysis of brain sections of single and double mutant animals (Figure 3). Consistent with previous results, the *Bmi1*^{-/-} cerebellums were significantly smaller than wildtype with all three layers of the cerebellum affected. We observed a similar level of cerebellum hypocellularity in the *E2f6*^{-/-};*Bmi1*^{-/-} mice. Given these observations, we conclude that E2F6-loss does not modulate either the defective cerebellar development or the consequent ataxia of the *Bmi1*-deficient mice.

It is well established that *Bmi1*-loss leads to the derepression of the *Ink4-Arf* locus and the resulting upregulation of p16^{INK4a} and p19^{ARF}. This upregulation is a key determinant of the hematopoietic and neural defects of the *Bmi1*^{-/-} mice via impairment of the self-renewal capacity of hematopoietic and neural stem cells (lessard and Sauvageau, 2003; Molofsky *et al.*, 2005; Molofsky *et al.*, 2003; Park *et al.*, 2003; Zencak *et al.*, 2005). The *Ink4-Arf* derepression was first observed, and is best characterized, in *Bmi1*^{-/-} MEFs where it causes impaired proliferation and premature senescence (Jacobs *et al.*, 1999). Given this fact, and the documented role of other E2F family members in the

Figure 3

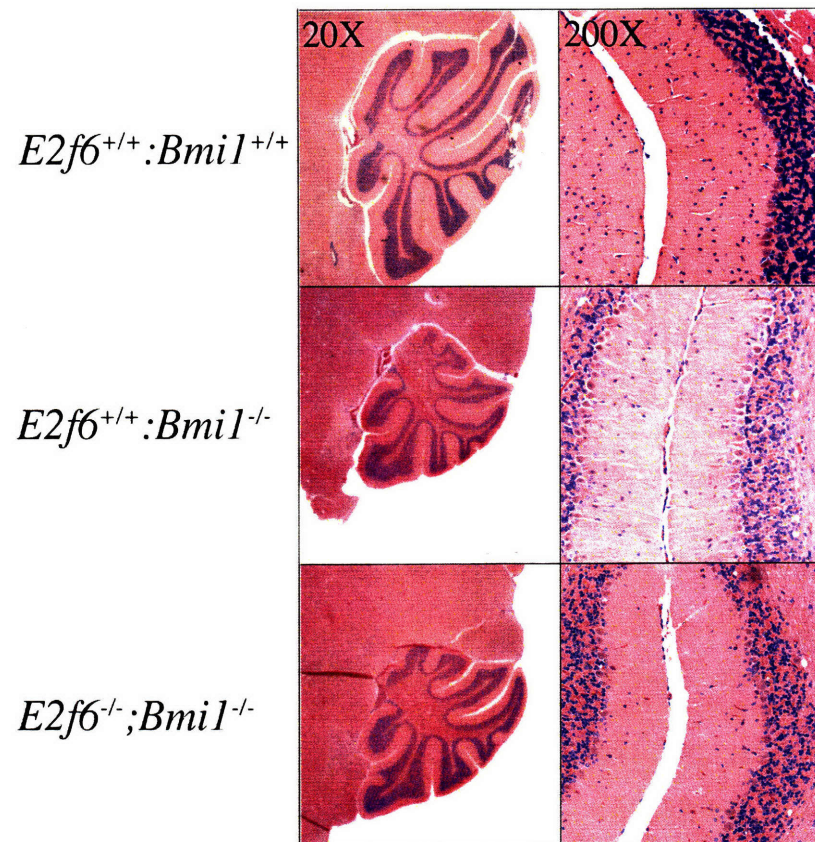


Figure 3: $E2f6^{-/-};Bmi1^{-/-}$ and $Bmi1^{-/-}$ mice display defects in gross cerebellar structure. Hematoxylin and eosin staining of cerebellum sections from the midline of 8 week old wildtype, $Bmi1$ mutant, and $E2f6$; $Bmi1$ compound mutant mice.

regulation of *Arf* in MEFs (Aslanian *et al.*, 2004), we also compared the properties of wildtype, *Bmi1*^{-/-}, *E2f6*^{-/-} and *E2f6*^{-/-};*Bmi1*^{-/-} MEFs. *E2f6*^{-/-} MEFs displayed no obvious proliferation defects (data not shown). In contrast, *Bmi1*^{-/-} MEFs showed a defect in asynchronous proliferation (Figure 4A) and underwent premature senescence (Figure 4B) as previously reported (Jacobs *et al.*, 1999). In addition, we found that serum deprived *Bmi1*^{-/-} MEFs were impaired in their ability to re-enter the cell cycle following the re-addition of serum (Figure 4C). There was some variation in the degree of these defects from one cell line to the next, likely because of the mixed genetic background. We also derived five *E2f6*^{-/-};*Bmi1*^{-/-} MEF lines from four different litters and compared their properties with those of MEFs derived from *Bmi1*^{-/-} (n=5) and wildtype (n=4) littermates. The presented data are from cell lines derived from one representative litter (Figure 4). The *E2f6*^{-/-};*Bmi1*^{-/-} MEFs showed defects in asynchronous proliferation, cellular senescence and cell cycle re-entry that were comparable to those of the *Bmi1*^{-/-} MEFs (Figure 4A-C). Consistent with this finding, real-time PCR analysis of RNA collected from these MEFs revealed the E2F6 status did not change the level of derepression of p16^{INK4a} and p19^{ARF} in the *Bmi1*^{-/-} MEFs (Figure 4D). Similarly, the levels of two E2F6 repressed genes, STAG3 and SMC1B, were not further derepressed in compound mutant MEFs (data not shown). These results suggest that E2F6 does not play a role in repression of *INK4a-ARF*.

Bmi1 and E2F6 synergize in axial skeleton development and co-regulate Hox genes.

To determine whether *E2f6* and *Bmi1* synergize in axial skeletal development, we examined the axial skeletons of all genotypes arising from a double heterozygous cross.

Figure 4

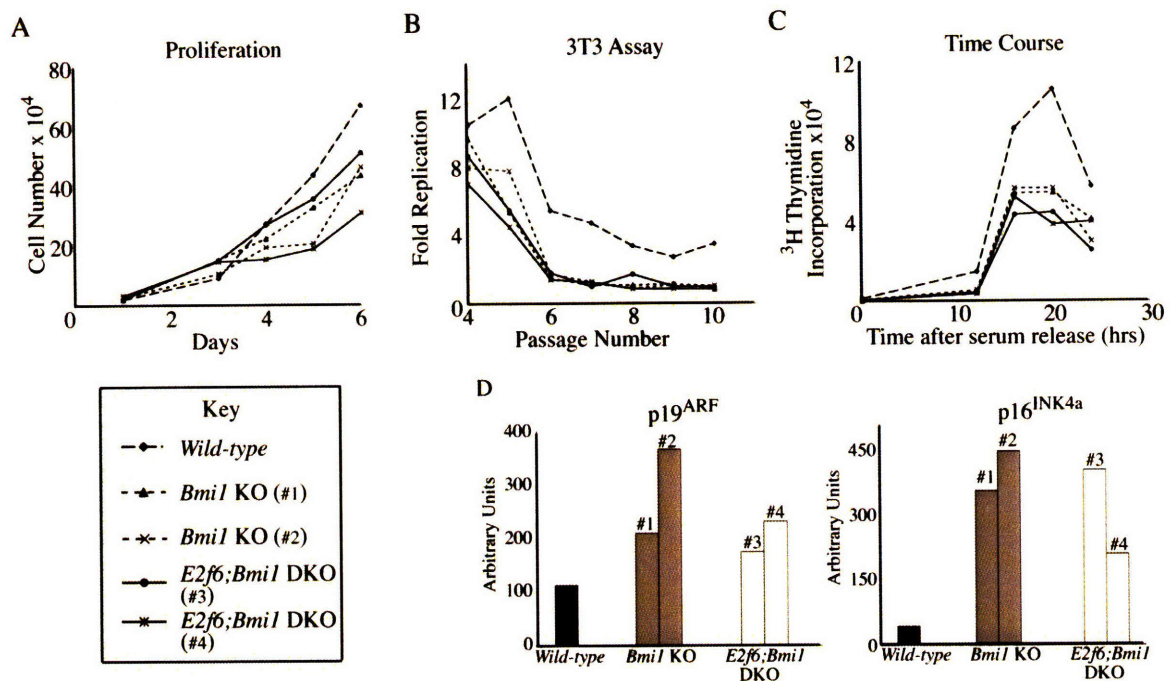


Figure 4: Cell cycle properties of *E2f6* and *Bmi1* mutant MEFs.

Mouse embryonic fibroblasts of wildtype (◆), *Bmi1* mutant (▲, ×), and *E2f6*; *Bmi1* compound mutant (*, ●) mice were assayed for (A) asynchronous proliferation rate, (B) senescence properties, (C) S-phase re-entry following serum withdrawal and re-addition, and (D) p16^{INK4a} and p19^{ARF} expression by RT-PCR from serum-starved cells.

Since 50% of the *Bmi1*^{-/-} mice die perinatally, we conducted this analysis using E18.5 embryos to ensure good representation of *Bmi1*^{-/-} and *E2f6*^{-/-};*Bmi1*^{-/-} animals (Figure 5). *Bmi1*^{-/-} mice are known to display morphological abnormalities along the anteroposterior axis that indicate posterior transformations of vertebra identity (van der Lugt *et al.*, 1994). These include (1) an extra piece of bone rostral to the cervical vertebra C1; (2) a C1 to C2 conversion; (3) a partial C7 to T1 (thoracic vertebra) conversion evidenced by the presence of ribs at C7, which then fuse on the ventral side with the ribs associated with T1 (in some cases, the transformation was full, that is, the C7 rib connected to the sternum instead of fusing with the T1 rib); (4) a T7 to T8 conversion resulting in only six instead of seven vertebrosteral ribs; (5) a T13 to L1 (lumbar vertebra) conversion shown by the absence of ribs at T13; and (6) an L6 to S1 (sacral vertebra) conversion evidenced by the joints between L6 and the iliac bones. Consistent with prior studies, we observed all of these transformations in the *Bmi1*^{-/-} embryos with partial penetrance (Figure 5). Analysis of the compound mutants showed that *E2f6* mutation increased the severity of the *Bmi1*^{-/-} skeletal defects in a dose dependent manner (Figure 5B). Specifically, deletion of only one allele of *E2F6* was sufficient to increase the penetrance of the C1 to C2, C5 to C6, partial C7 to T1, T13 to L1, and L6 to S1 transformations. Further deletion of the remaining *E2f6* allele led to an even higher penetrance, indicating that *E2F6* and *Bmi1* synergistically contribute to these abnormalities. No synergy was found for the extra, C7 to T1 (full), and T7 to T8 transformations, suggesting that these defects are specific to *Bmi1*-loss. However, in the case of the extra piece of bone (E) we observed an increase in the *E2f6*^{+/-};*Bmi1*^{-/-} but not in the *E2f6*^{-/-};*Bmi1*^{-/-} mice. As this piece of bone is very small and weakly attached, we cannot rule out the possibility that it was broken off during

Figure 5

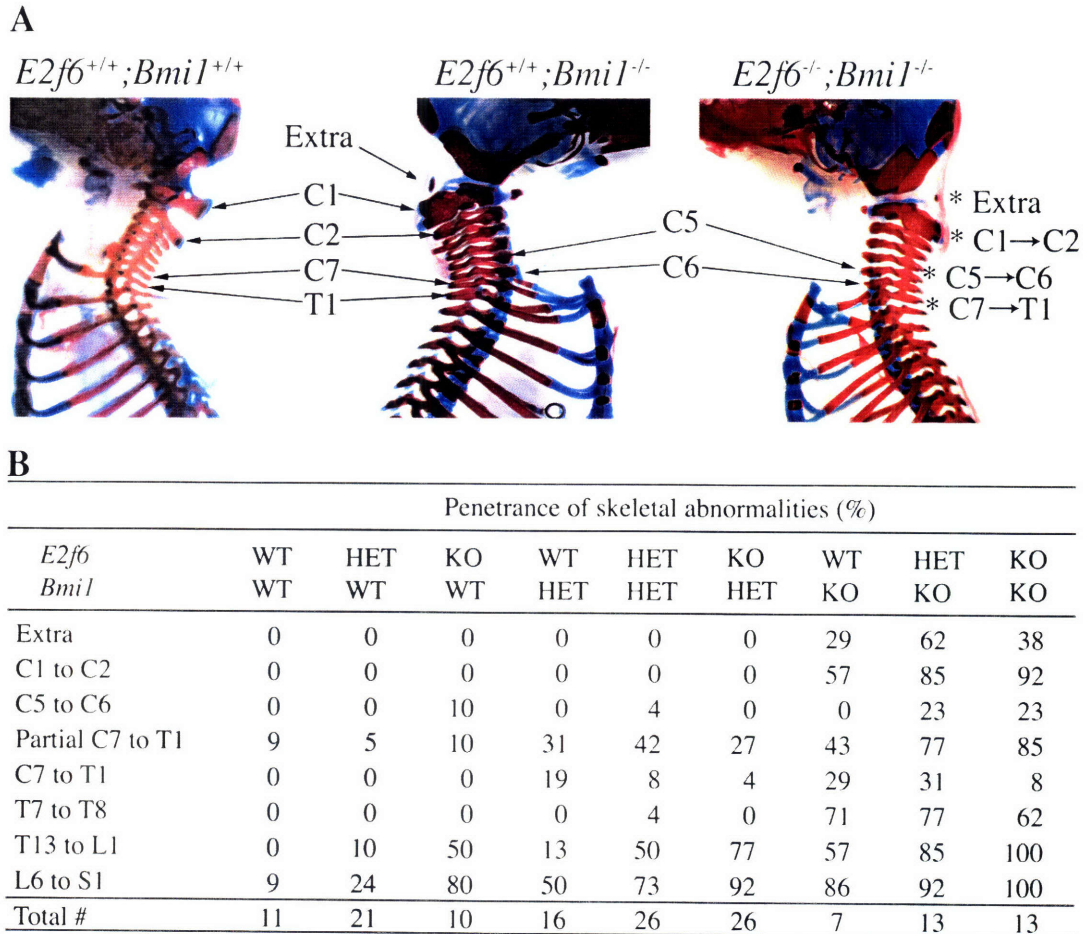


Figure 5: *E2f6* and *Bmi1* show a genetic interaction in the development of the axial skeleton.

(A) Alcian blue (cartilage) and alizarin red (bone) stainings of wildtype, *Bmi1* mutant and *E2f6*;*Bmi1* compound mutant mice at E18.5. The following skeletal transformations are depicted in the *Bmi1* and *E2f6*;*Bmi1* compound mutant mice: An extra piece of bone anterior to the first cervical vertebra (C1); the C1 vertebra is transformed into the second cervical vertebra (C2) as evidenced by the altered morphology; the seventh cervical vertebra (C7) is transformed into the first thoracic vertebra (T1) as shown by the presence of vertebrosteral ribs. The right panel shows the novel C5 to C6 transformation present in the *E2f6* mutant and *E2f6*;*Bmi1* compound mutant mice. This is evidenced by the presence of a piece of cartilage on C5 instead of on C6. (B) Penetrance of axial skeletal transformations in *E2f6*;*Bmi1* compound mutant embryos.

manipulation of the axial skeletons. Finally, we have identified a novel transformation, the C5 to C6 conversion that occurs in 10% of *E2f6*^{-/-} embryos and 23% of *E2f6*^{-/-};*Bmi1*^{-/-} embryos but was never detected in the *Bmi1*^{-/-} embryos. The increase in penetrance of some, but not all, posterior transformations in the *E2f6*^{-/-};*Bmi1*^{-/-} embryos underscores the conclusion that E2F6 influences the regulation of a subset of *Bmi1* target genes.

It is well established the axial skeletal defects in the *Bmi1* mutants results from the derepression of *Hox* genes that are essential for embryonic patterning. The observed synergy between *E2f6* and *Bmi1* in axial skeletal development suggests that E2F6 and *Bmi1* might co-regulate *Hox* genes. To address this question, we first used real-time PCR analysis to compare *Hox* mRNA levels genes in the wildtype (n=2), *Bmi1*^{-/-} (n=2), and *E2f6*;*Bmi1* DKO (n=3) MEFs used above to assess *Ink4a-Arf* expression. Although E2F6-loss had no effect on the level of derepression of p16^{INK4a} and p19^{ARF} in the *Bmi1*^{-/-} MEFs (Figure 4D), it did modulate the *Bmi1* mutant phenotype with regard to the *Hox* genes (Figure 6A). The *Bmi1*^{-/-} MEFs had increased mRNA levels of *HoxC10* (both lines analyzed), *HoxA9* (both lines analyzed) and *HoxB6* (1 out of 2 lines analyzed). Loss of E2F6 in the *Bmi1* mutant cells led to further deregulation of *HoxB6* and *HoxC10*, but not *HoxA9*, in every *E2f6*;*Bmi1* mutant cell line analyzed (Figure 6A and data not shown). The mRNA levels of *HoxB6*, *HoxC10*, or *HoxA9* were unaffected by the mutation of *E2f6* alone (data not shown) indicating that this reflects a synergistic effect of *E2f6* and *Bmi1* in *Hox* gene regulation.

The MEF analysis supports our genetic evidence that *E2f6* and *Bmi1* act together to regulate *Hox* genes but not *Arf*. However, these experiments do not establish whether E2F6 is directly, or indirectly, involved in the transcriptional regulation of the *Hox* genes.

Figure 6

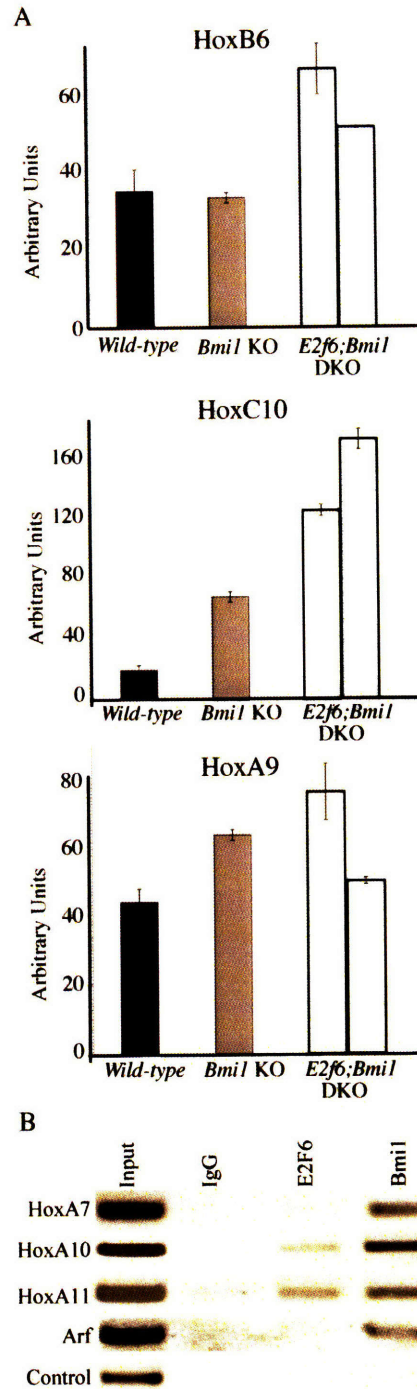


Figure 6: E2f6 and Bmi1 co-regulate *Hox* genes.

(A) Real-time PCR analysis of *Hox* genes in MEFs. (B) Chromatin immunoprecipitation analysis of mouse embryonic stem cells. Sonicated, cross-linked chromatin was immunoprecipitated with a Bmi1, E2F6, or control IgG antibody and the purified DNA was analyzed by PCR with primers specific for the promoter of *HoxA7*, *HoxA10*, *HoxA11*, *Arf*, or a control sequence (1kb upstream of the E2F1 promoter).

To address this question, we wished to evaluate the promoter occupancy of the *Hox* genes. It has previously been shown that repression of *Hox* genes and other key developmental regulators is established in embryonic stem (ES) cells (Boyer *et al.*, 2006). Thus, we used murine ES cells to perform chromatin immunoprecipitation on four genes, *HoxA7*, *HoxA10*, *HoxA11*, and *Arf*, which have previously been shown to be directly regulated by Bmi1 (Bracken *et al.*, 2007; Cao *et al.*, 2005; Kotake *et al.*, 2007; Xi *et al.*, 2007). We found that Bmi1 was directly bound to the promoters of *HoxA7*, *HoxA10*, *HoxA11*, and *Arf* in mouse embryonic stem cells (Figure 6B). In contrast, E2F6 bound specifically to the promoters of *HoxA10* and *HoxA11*, but did not associate with either *HoxA7* or *Arf* (Figure 6B). These results provide *in vivo* biochemical evidence of the co-regulation of a subset of *Hox* genes by E2F6 and Bmi1 and reinforce the conclusion that E2F6 is not required for repression of *Arf*.

DISCUSSION

Bmi1 is a key component of the PRC1 repressor complex. *Bmi1* mutant mice have axial skeletal transformations that reflect a key role for Bmi1 in the appropriate repression of the *Hox* genes (Akasaka *et al.*, 1996; Akasaka *et al.*, 1997; del Mar Lorente *et al.*, 2000; van der Lugt *et al.*, 1994). Additionally, *Bmi1*-deficient mice have impaired proliferation and premature senescence due to the upregulation of *Ink4a-Arf* (Jacobs *et al.*, 1999). Bmi1 loss and upregulation of p16^{INK4a} and p19^{ARF} compromises the proliferative and self-renewal capacity of stem cells in the developing mouse (Lessard and Sauvageau, 2003; Molofsky *et al.*, 2003; Park *et al.*, 2003). This causes impaired development of both the hematopoietic compartment and nervous system of *Bmi1* mutant

mice. Consequently, these mice display reduced newborn survival, ataxia, anemia, and a reduction of cell populations in the hematopoietic compartment (van der Lugt *et al.*, 1994). The neural stem cell defect can be partially rescued by disruption of the *Ink4a-Arf* locus (Molofsky *et al.*, 2005). Although Bmi1 has been shown by chromatin immunoprecipitation to be present at the promoters of p16^{INK4a} and p19^{ARF} (Bracken *et al.*, 2007; Kotake *et al.*, 2007), Bmi1 is unable to directly bind to DNA in a sequence-specific manner (Alkema *et al.*, 1997; Tagawa *et al.*, 1990).

We have previously established that E2F6 associates with Bmi1 and other known components of the PRC1 complex *in vivo* (Trimarchi *et al.*, 2001). Since other members of the E2F family are known to regulate *Arf*, we hypothesized that E2F6 might cooperate with Bmi1 in the regulation of the *Ink4a-Arf* locus, and perhaps also in axial skeletal development. To address this question, we have generated *E2f6* mutant mice and subsequently *E2f6;Bmi1* compound mutants. Despite the documented role of various E2F family members in the regulation of *Arf* (Aslanian *et al.*, 2004), we did not detect any evidence that E2F6 contributes to the regulation of the *Ink4a-Arf* locus. First, E2F6-loss had no detectable effect on the proliferation properties of MEFs and there was no detectable derepression of p19^{ARF}. Second, we observed no further derepression of p16^{INK4a} and p19^{ARF} in *E2f6*^{-/-};*Bmi1*^{-/-} versus *Bmi1*^{-/-} MEFs and no significant difference in the proliferative or senescent properties of these two genotypes. Finally, E2F6 was not detected at the promoter of *Arf* in ES cells. We did find that Bmi1 deficient cells have a cell cycle re-entry defect that has not been previously reported. However, the compound mutant cells do not differ significantly in their ability to re-enter the cell cycle.

Previously, E2F4 had been shown to compensate for loss of E2F6 at the promoters of cell

cycle genes (Zhu *et al.*, 2004), and one hypothesis was that a lack of a genetic interaction between E2F6 and Bmi1 could be due to E2F4 or another E2F family member compensating for the loss of E2F6 at *Arf*. However, the promoter occupancy analysis presented here strongly suggests that E2F6 plays little or no role in the direct regulation of *Ink4a-Arf*. Certainly, our data show that E2F6 is fully dispensable for the appropriate regulation of this locus. Consistent with this finding, the loss of E2F6 did not exacerbate the defects in either the hematopoietic compartment or the cerebellar development of the Bmi1 mutant mice that largely result from the derepression of *Ink4a-Arf*. Thus, these data suggest that E2F6 does not influence the regulation of *Ink4a-Arf* by Bmi1 by either direct or indirect mechanisms.

In stark contrast to the regulation of *Ink4a-Arf*, our data show a role for *E2f6* in axial skeletal development. First, data from both this and previous (Storre *et al.*, 2002) studies show that, like other PcG proteins, E2F6-loss results in axial skeletal transformations. Second, we find that *E2f6* mutation acts in a dosage-dependent manner to increase the penetrance of skeletal transformations in the *Bmi1* deficient background. This genetic analysis suggests that E2F6 plays a vital role in the regulation of a subset of Bmi1 target genes, presumably the *Hox* genes. To further this study, we took a more biochemical approach to analyze if the increased penetrance of the skeletal transformations in the *E2f6;Bmi1* mutant mice is due to enhanced deregulation and loss of direct transcriptional control of the *Hox* genes. Indeed, we did find enhanced deregulation of a subset of Bmi1 target *Hox* genes and a direct association of E2F6 with a subset of Bmi1-responsive *Hox* gene promoters. This biochemical analysis clearly provides insight into how E2F6 loss in *Bmi1* mutant mice leads to an increase in the

penetrance of skeletal transformations. Furthermore, it underscores the diversity of the Bmi1 complexes and the mechanisms by which it regulates target genes. Finally, we have established that E2F6 directly regulates *Hox* genes *in vivo* and have reported a novel C5 to C6 transformation which is present in *E2f6* and *E2f6;Bmi1* mutant embryos. Thus, we conclude that E2F6 and Bmi1 act synergistically in development of the axial skeleton. Taken together, our data show that E2F6 plays a vital role in the regulation of a subset of Bmi1 target *Hox* genes that govern the anteroposterior patterning of the developing embryo but is not required for the regulation of *Ink4a-Arf* and the control of cellular proliferation.

EXPERIMENTAL PROCEDURES

Generation and genotyping of *E2f6*^{-/-} mice

The BAC clone b39J22 (Research Genetics) known to contain the *E2f6* genomic locus was mapped and cloned to obtain the sequences necessary for designing the targeting strategy. The targeting vector described above was introduced into 129Sv J1 ES cells by electroporation and the cells were selected with G418 and Gancyclovir. Ninety-six resistant clones were picked for genotyping. *E2f6*^{+/-} cells were detected by Southern blot using external 5' and 3' probes as well as a neomycin probe. Once the heterozygous clones were identified and verified to contain a diploid genome by karyotyping, they were injected into C57/BL6 3.5 d.p.c. blastocysts. The injected blastocysts were subsequently implanted into pseudo-pregnant females and the chimeric progeny were identified by coat color. Mice with a high contribution of agouti cells were

mated to pure C57/BL6 mice. The agouti progeny of these mice were genotyped by PCR of DNA obtained from ear or tail pieces using the common primer 5'-ATCTCTGTCTGGTCTGATCC-3', the wildtype *E2f6*-specific primer 5'-GATGCCATCCAAGACATTGG-3', and the mutant targeting vector specific primer 5'-GCCGCATAACTTCGTATAGC-3'. The *E2f6*^{+/-} mice were then interbred to produce *E2f6*^{-/-} mice.

Histological and skeletal analysis

Euthanized animals were dissected and processed for histological analysis. Soft tissues were fixed in 10% formalin and hard tissues were fixed in Bouin's fixative. Paraffin sections were prepared and stained with hematoxylin and eosin. Skeletal analysis was performed on 3-day old mice and 18.5 d.p.c. embryos. After removing the skin and viscera, the skeletons were fixed in acetone and stained with cartilage-specific Alcian Blue and bone-specific Alzarin Red. Soft tissue was cleared with KOH.

Mouse embryonic fibroblasts and cell cycle assays

MEFs were prepared from 13.5 d.p.c. embryos as previously described (Humbert *et al.*, 2000) and genotyped by PCR of DNA obtained from yolk sacs. Proliferation curves were obtained by plating 2×10^4 MEFs in triplicate in 24-well plates. At the indicated time points, MEFs were trypsinized and counted. For cell cycle re-entry assays, 2×10^5 MEFs were plated in triplicate in 6-well plates. After 2 days of growth in media containing 10% serum, cells were incubated in media containing 0.1% serum for 3-4 days. Re-entry into the cell cycle was induced by incubation in media containing 10% serum and at the indicated time points, 5 μ Ci of ³H-thymidine was added to the cells for 1 hour. Cells were then scraped from the plates and cell pellets were analyzed for ³H-

thymidine incorporation using a scintillation counter. A 3T3 protocol was followed to monitor senescence. 3×10^5 MEFs were plated in duplicates in 6-cm plates and re-fed 2 days later. On the third day, they were trypsinized, counted and replated. The fold replication was determined by dividing the number of cells obtained at day 3 by 3×10^5 . Western blots were performed as described previously (Moberg *et al.*, 1996) with 50-100 μ g of whole cell lysates using primary antibodies against E2F6 (mouse monoclonal, clone 2E10, J.A. Lees) and p19^{ARF} (rat monoclonal, sc-32748, Santa Cruz Biotechnology).

Real-Time PCR Analysis

For RT-PCR analysis, RNA was collected from asynchronously proliferating cell pellets. RNA was processed using the Rneasy MinElute Cleanup Kit (Qiagen, 74204). cDNA was made from the RNA using SuperScript First-Strand Synthesis System (Invitrogen, 11904-018). 2 μ l of cDNA (diluted 1:100), 0.9 μ M primer pair, and 10 μ l of SybrGreen Master Mix (Applied Biosystems, 4309155) was used for each PCR reaction. RT-PCR signals were normalized to an ubiquitin internal control. Primer sequences are available upon request.

Analysis of the hematopoietic compartment

Single-cell preparations were made from the bone marrow, thymus, and spleen of eight-week old mice by mincing the tissue and then pressing through a nylon mesh. Cells were then counted with a hemocytometer. For flow cytometry analysis, cells were resuspended in a 96-well plate at 3×10^5 cells/well in FACS buffer (PBS, 0.5% BSA, 0.1% NaN₃). Cells were washed with FACS buffer, blocked with Fc Block (1/4000 in FACS buffer, BD Biosciences), and incubated with saturating amounts of monoclonal antibodies conjugated to FITC, PE, or Biotin at 4°C for 30 minutes in the dark. Cells

were then washed 2 times with FACS buffer and, if required, incubated with a secondary antibody (Streptavidin-APC) at 4°C for 30 minutes in the dark. Cells were then washed 2 times with FACS buffer and resuspended in 400 μ l PI-containing FACS buffer (1 μ g/ml). Cells were then analyzed on a FACScan. The following antibodies were used: Anti-mouse IgM-Biot (IB4ABI, Southern Biotechnology Associates), PE-Anti-Mouse CD8a (553032, BD Pharmingen), Biotin Anti-Mouse CD4 (553044, BD Pharmingen), PE Anti-mouse B220 (553089, BD Pharmingen), PE Anti-mouse Ly-6G (Gr-1) (553128, BD Pharmingen), Biotin Anti-mouse CD11b (Mac-1) (557395, BD Pharmingen), Streptavidin-APC (554067, BD Pharmingen), FITC Anti-mouse CD28 (IL2-R) (553071, BD Pharmingen), Biotin Anti-mouse CD24 (HSA) (555296, BD Pharmingen).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed as previously described (Aslanian *et al.*, 2004). Oligonucleotide primers (Integrated DNA Technologies) used for PCR analysis are available upon request. Antibodies used were E2f6 (sc-8366, Santa Cruz Biotechnology) and Bmi1 (Bmi1-1, J.A. Lees).

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Chapter 3

E2f6 loss suppresses gliosis after neuronal injury

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ABSTRACT

Astrocyte activation or gliosis occurs in response to neurodegeneration and neuronal death. Upon CNS insult, astrocytes become activated and form a glial scar around the damaged area. This process provides neuronal protection by restricting inflammation and regulating the concentration of molecules in the extracellular environment. However, gliosis has potentially detrimental effects such as the inhibition of axonal regeneration or the release of cytotoxic agents that trigger degeneration of neighboring neurons. The molecular mechanisms required to initiate and sustain a gliotic response are poorly understood. This analysis describes a novel requirement for E2F6 in gliosis. Upon neural injury, the gliotic response of *E2f6*^{-/-} mice is suppressed. This phenotype is not due to a modulation of apoptosis or proliferation, suggesting that *E2f6* loss uncouples cell damage from gliosis.

INTRODUCTION

The E2F transcription factors act by controlling the transcription of genes that are essential for cell cycle progression and DNA synthesis (Dyson, 1998; Nevins, 1998; Trimarchi and Lees, 2002). In mammals, eight *E2f* genes (*E2f1-8*) have been identified. *E2f1-5* encode proteins that function as transcriptional activators or repressors by virtue of their association with the family of pocket proteins. *E2f6-8* are more distantly related members of the E2F family. In particular, they lack the domain required for pocket protein-binding, and these E2Fs are not susceptible to pocket protein regulation (Morkel *et al.*, 1997; Cartwright *et al.*, 1998; Trimarchi *et al.*, 2001; de Bruin *et al.*, 2003; Logan *et al.*, 2004). E2F6 binds DNA as a heterodimer with DP, in a similar manner to E2F1-5. Due to the absence of a transactivation domain, it does not activate transcription (Morkel *et al.*, 1997; Cartwright *et al.*, 1998; Gaubatz *et al.*, 1998; Trimarchi *et al.*, 1998). The repressive functions of E2F6 are achieved through its interactions with chromatin modifiers and the polycomb group family of proteins (Trimarchi *et al.*, 2001; Ogawa *et al.*, 2002; Attwooll *et al.*, 2005). The polycomb group proteins are repressors of homeobox (*Hox*) genes, which control the anterior-posterior patterning of the developing embryo.

In vivo mouse models have revealed novel roles for E2Fs in different developmental processes. To uncover the roles of E2F6 in development, we and others have generated mice deficient for this gene (Storre *et al.*, 2002; Courel *et al.*, 2008). These mice are viable, and they live a normal life-span. *In vitro* studies did not reveal any cell cycle or proliferation defects. *E2F6* mutant mice, however, exhibit a defect in spermatocyte development and posterior transformations of the axial skeleton (Storre *et*

al., 2002; Courel *et al.*, 2008). The axial skeleton transformations seen in these mice reflect E2F6's role in *Hox* gene regulation. In this study, we describe a novel role for E2F6 in reactive gliosis.

Reactive gliosis refers to cellular changes that astrocytes undergo following brain trauma. Activation of astrocytes is characterized by an increase in the size of the cell body and processes and an increase in the levels of an intermediate filament protein called GFAP (glial fibrillary acidic protein). During both normal brain function and trauma, astrocytes play a vital role in scavenging potentially toxic agents from the extracellular matrix. After acute neuronal damage to the central nervous system, there is a release of glutamate from neurons (Swanson *et al.*, 2004). Glutamate and potassium uptake by astrocytes prevents toxic elevations of these molecules in the brain and extracellular space (Aschner and Kimelberg, 1991; Anderson and Swanson, 2000). Astrocytes also contain the highest concentration of antioxidants and are hypothesized to protect neurons from oxidative stress following neurodegeneration (Sano *et al.*, 1997; Marcus *et al.*, 1998; Dringen, 2000; Dringen and Gutterer, 2002). Astrocytes have acute neuroprotective properties that could potentially relieve stress on neurons after induction of neurodegeneration or injury to the brain.

There is an increasing amount of evidence that gliosis may exacerbate the pathologies of individuals with neurodegenerative disease and may impede neural repair. Inhibiting gliosis in mice with neurodegeneration disorders or ischemia improves the pathology of these mice. In a mouse model for Alzheimer's disease, anti-inflammatory drugs such as ibuprofen have been shown to suppress the gliotic response and reduce amyloid plaque formation (Lim *et al.*, 2000; Jantzen *et al.*, 2002; Yan *et al.*, 2003).

Similarly, dispensing ibuprofen to mice or rats subjected to ischemia reduces neuronal injury and improves cerebral blood flow and neurological outcome (Kuhn *et al.*, 1986; Cole *et al.*, 1993; Patel *et al.*, 1993; Antezana *et al.*, 2003). These studies suggest that blocking the gliotic response in individuals with neurodegeneration may be a successful therapeutic option. Little is known about the molecular mechanisms contributing to gliosis *in vivo*. There is some suggestion that neural injury induced by neurodegenerative diseases, tumorigenesis, and ischemia initiates inappropriate activation of a program required for development of the central nervous system (Liu *et al.*, 2006). This model explains the duality of gliosis and suggests that this early developmental program is harmful to neurons in adult individuals.

Our analysis of *E2f6* mutant mice did not reveal any defects in the central nervous system. However, we did find that these mice exhibit a suppression of gliosis when cell damage is induced with either the injection of a neurotoxin (methylazoxy methanol) or the loss of *Bmi1*. *Bmi1* loss has been shown to induce apoptosis of cells in the cerebellum and cortex of mice. Following cell death, *Bmi1* mutant mice demonstrate a gliotic response. Eight-week old mice deficient for both *E2f6* and *Bmi1* fail to undergo gliosis. To determine whether this defect in gliosis is specific to *Bmi1* loss or whether it occurs upon the induction of other types of brain injury, we used methylazoxy methanol to induce brain damage in mice deficient for *E2f6*. Under these conditions, *E2f6* mutant mice also display a decrease in gliosis. This defect in gliosis was not due to a loss of proliferation or a reduction of apoptosis. These results suggest that E2F6 plays a vital role in the induction of gliosis.

RESULTS

***E2f6* loss suppresses gliosis in the cerebellum of eight-week old *Bmi1* mutant mice.**

Bmi1 loss in the cerebellum and cortex induces a gliotic response characterized by an increase in reactive astrocytes in the molecular layer of the cerebellum and an increase in GFAP immunoreactivity in activated astrocytes. This response is most pronounced in the Bergmann's glia in the cerebellum of these mice (Figure 1). As expected, all eight-week-old wildtype or *E2f6* mutant mice analyzed did not exhibit gliosis in the absence of any brain injury. Gliosis was seen in the cerebellum in five out of seven eight-week-old *Bmi1* mutant mice. No apoptosis was observed (data not shown), suggesting that the gliosis seen at this time-point is better characterized as a "glial scar" resulting from neonatal apoptosis in the cerebellum. In all eight-week-old mice deficient for both *E2f6* and *Bmi1* the gliotic response is greatly suppressed (n=5), and this is accompanied by a reduction in the number of astrocytes seen in the molecular layer of the cerebellum (Figure 1). These results suggest that E2F6 is required for the gliotic response induced by the loss of *Bmi1*.

***E2f6* loss suppresses gliosis induced by methylazoxymethanol, a neurotoxin.**

E2F6 and *Bmi1* interact biochemically (Trimarchi *et al.*, 2001), and participate in the transcriptional repression of some of the same *Hox* genes (Courel *et al.*, 2008). For this reason, it is unclear if the suppression of gliosis seen in mice deficient for both *E2f6* and *Bmi1* is due to loss of a repressive complex containing both proteins or if this suppression is independent of *Bmi1* loss. To test this, we used methylazoxymethanol

Figure 1

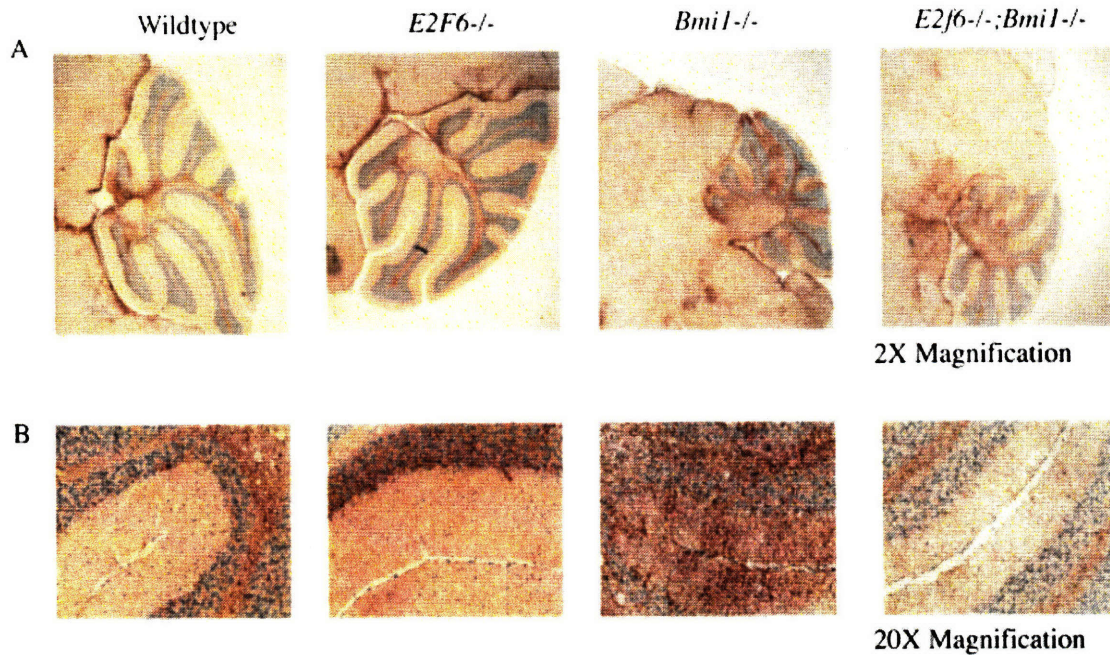


Figure 1: GFAP immunohistochemistry of eight-week-old mice.

GFAP immunohistochemistry marks activated astrocytes and gliosis after brain injury. (A) Low magnification picture of the cerebellums of eight-week-old wildtype, *E2f6* mutant, *Bmi1* mutant, and *E2f6*;*Bmi1* compound mutant mice. (B) High magnification of the molecular and granule layer of eight-week-old wildtype, *E2f6* mutant, *Bmi1* mutant, and *E2f6*;*Bmi1* compound mutant mice. Pronounced GFAP immunoreactivity is seen in the molecular layer of *Bmi1* mutant mice.

(MAM), to induce gliosis in mice mutant for only *E2f6*. MAM, similar to *Bmi1* loss, inhibits the proliferation of cells in the external granule layer of the cerebellum and causes apoptosis of these cells. Thus, this drug should recapitulate *Bmi1* loss. MAM was administered to three-day-old pups obtained from an intercross of *E2f6*^{+/-} mice. Two days later, the pups were sacrificed and sectioned for histological analysis. All wildtype pups (n=12) exhibited a proper gliotic response. In contrast, gliosis was suppressed in five out of seven *E2f6*^{-/-} mice (Figure 2). Thus, the suppression of gliosis by E2F6 is partially penetrant and independent of *Bmi1* loss.

The E2F transcription factors are known to control apoptosis and proliferation of primary cells. Specifically, E2F6 has been shown to modulate the apoptotic response in human embryonic kidney 293 cells in response to ultraviolet-induced apoptosis (Yang *et al.*, 2007). However, there was no modification of apoptosis or proliferation in the cerebellums of wildtype *verses* *E2f6*^{-/-} pups injected with MAM (Figure 3). In conclusion, *E2f6* loss causes a partially penetrant reduction in gliosis that is not due to a modulation of apoptosis or proliferation.

***E2f6* does not inhibit gliosis in five-day-old *Bmi1* mutant neonates.**

In order to better characterize the suppression of gliosis by E2F6, we analyzed *Bmi1*;*E2f6* compound mutant mice at five-days-old. Surprisingly, *E2f6* loss did not suppress gliosis in this setting (n=5; Figure 4). One hypothesis is that there is a threshold level of gliosis suppressed by *E2f6* loss. This hypothesis may also explain the partially penetrant phenotype observed in the pups injected with MAM.

Figure 2

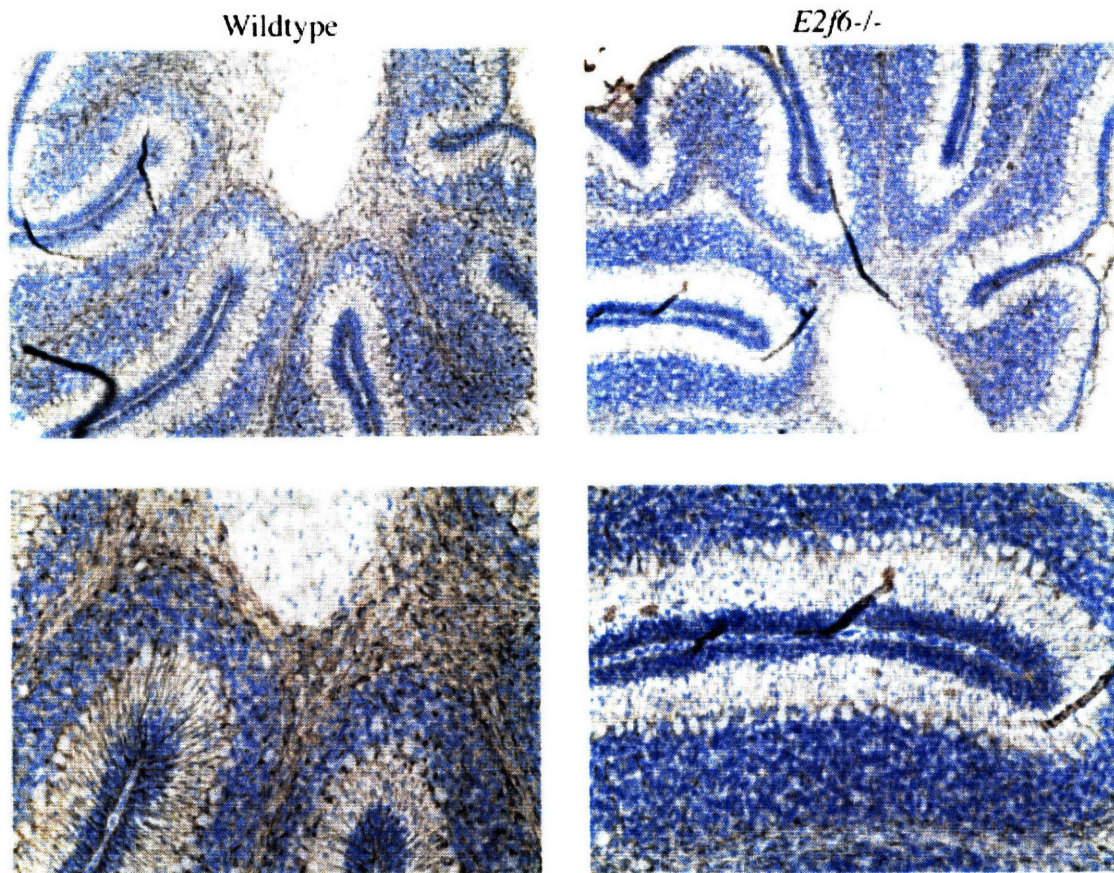


Figure 2: GFAP immunohistochemistry of postnatal day 5 mice injected with MAM. Postnatal day 3 pups obtained from *E2f6*^{+/-} intercrosses were injected with methylazoxymethanol. Two days later, pups were sacrificed and brain tissues were collected.

Figure 3

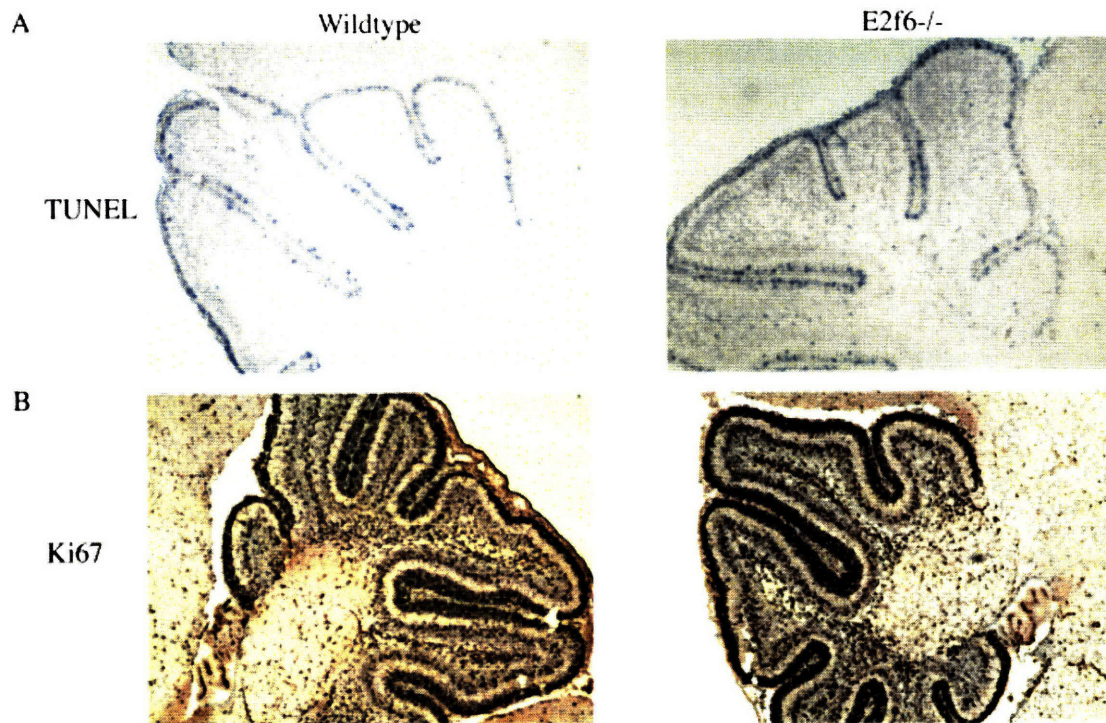


Figure 3: Analysis of apoptosis and proliferation of MAM injected pups.

Postnatal day 3 pups obtained from *E2f6*^{+/-} intercrosses were injected with methylazoxymethanol. Two days later, pups were sacrificed and brain tissues were collected. Tissues were analyzed by (A) TUNEL staining for apoptosis and (B) Ki67 immunohistochemistry for proliferation.

Figure 4

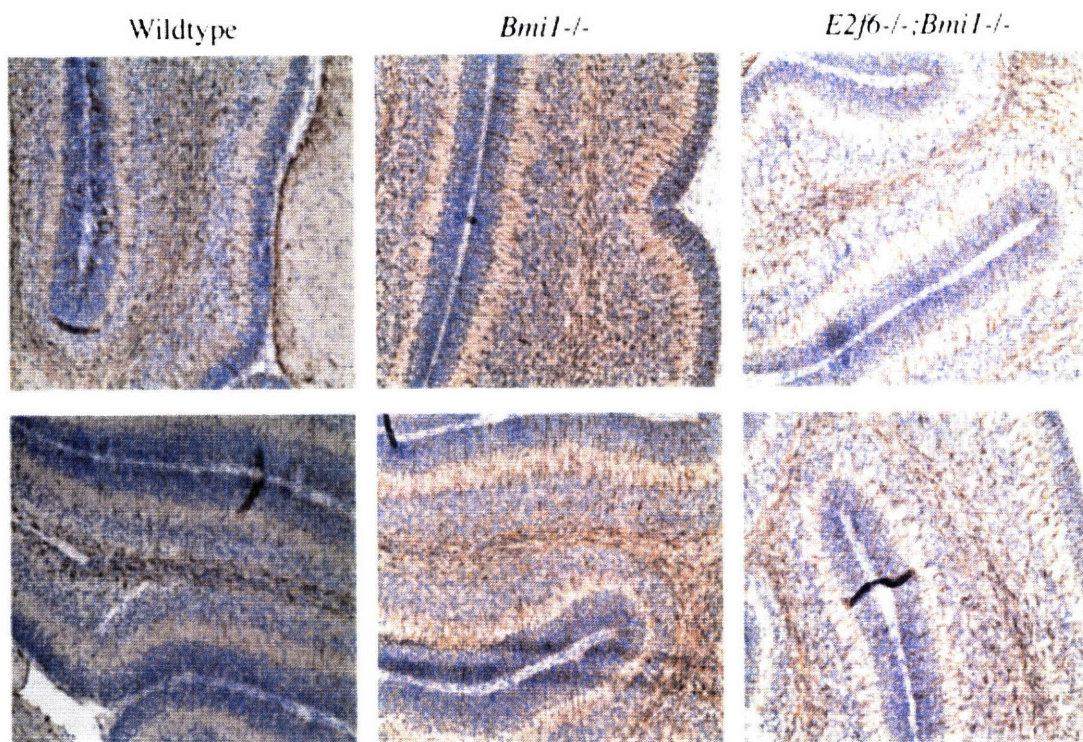


Figure 4: GFAP immunohistochemistry of five-day-old mice.

GFAP immunohistochemistry was performed on wildtype, *Bmi1* mutant, and *E2f6*;*Bmi1* compound mutant mice.

DISCUSSION

There is mounting evidence that gliosis contributes to pathogenesis of neurodegenerative diseases. Blocking this process in mouse models for Alzheimer's and ischemia improves the pathology of these mice (Kuhn *et al.*, 1986; Cole *et al.*, 1993; Patel *et al.*, 1993; Lim *et al.*, 2000; Jantzen *et al.*, 2002; Antezana *et al.*, 2003; Yan *et al.*, 2003). For this reason, it is not surprising that many therapies for neuronal injury focus on blocking the gliotic response. Little is known about the molecular mechanisms involved in triggering or sustaining a proper gliotic response.

In this study, we present a novel role for E2F6 in gliosis. Gliosis is suppressed in eight-week-old *E2f6* mutant mice with neuronal injury induced by *Bmi1* loss and five-day-old *E2f6* mutant pups injected with a neurotoxin (MAM). In *E2f6*^{-/-} pups injected with MAM, the phenotype was partially penetrant, with five out of seven *E2f6*^{-/-} pups showing a suppression of gliosis. Gliosis was not suppressed in five-day-old *E2f6*^{-/-} pups deficient for *Bmi1*. We hypothesize that there is a threshold level of gliotic activity that *E2f6* loss can suppress. To further investigate these results, it would be interesting to do a whole-genome analysis of mRNA changes in brain extracts of wildtype and *E2f6* mutant mice subjected to brain injury. Finally, it will be important to investigate whether *E2f6* loss modulates the gliosis and neurodegenerative phenotypes of mice with Alzheimer's disease.

EXPERIMENTAL PROCEDURES

Immunohistochemistry

Slides were dewaxed and rehydrated. Antigen unmasking was done by boiling slides for 15 minutes in 10mM Sodium Citrate/0.05% Tween, pH6. Slides were cooled for 15 minutes at room temperature and then washed in water three times for five minutes each wash. To block endogenous peroxidases, slides were blocked in 1% hydrogen peroxide for 20 minutes, washed with water 3 times for five minutes each wash, and PBS one time for five minutes. Next was a blocking step with 5% goat serum in PBS for 1 hour at room temperature. The primary antibody GFAP (DakoCytomation, Z0334, Rabbit polyclonal, diluted 1:1000) or Ki67 (BD biosciences, 347580 and 550609, diluted 1:50) was diluted in blocking solution (5% goat serum in PBS) and incubated overnight at 4°C. The following day, slides were washed three times in PBS for five minutes each wash. Secondary antibody (Vectastain ABC kits, vector laboratories) was diluted 1:500 in block, and slides were incubated with the secondary antibody for 1 hour at room temperature. A DAB substrate was used for the detection per manufacturer's instructions (Vector laboratories). Following the detection, slides were counterstained with Harris Hemotoxin and mounted using standard protocols.

TUNEL Staining

TUNEL staining was performed using a In Situ Cell Death Detection Kit, AP (Roach, 11684809910). Slides were dewaxed. An antigen retrieval step was performed by boiling the slides in 10mM Sodium Citrate/0.05% Tween, pH6 for fifteen minutes. TUNEL staining was performed per manufacturer's instructions (Roach, 11684809910).

Methylazoxy methanol acetate injections

Mice at postnatal day three were injected subcutaneously with 43mg/kg of MAM (NCI Repository, NCI# F0040, MRI# 213) dissolved in physiological saline at 2mg/ml. A maximum volume of 100 microliters was injected. Two days later, mice were euthanized by carbon dioxide inhalation followed by either decapitation or opening of the thorax. Brain tissues were collected for histological analyses. The cage bedding and carcasses were handled as biohazardous materials, and the cage was washed with sodium bicarbonate.

Acknowledgement of Source

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Chapter 4

Discussion

E2F6 is a repressor of transcription (Morkel et al., 1997; Gaubatz et al., 1998; Trimarchi et al., 1998) that has been shown to biochemically interact with members of the mammalian polycomb group family of proteins (Attwooll et al., 2004). Little is known about the biological role of E2F6 in development and polycomb repression. Thus, to investigate E2F6's role *in vivo*, we generated mice deficient for *E2f6*. *E2f6* mutant mice display subtle axial skeletal transformations. These include the T13-L1 and L6-S1 posterior axial skeletal transformations (Storre et al., 2002; Courel et al., 2008). MEFs isolated from *E2f6*^{-/-} mice do not have any proliferation or cell cycle defects (Storre et al., 2002). Compound mutants of polycomb group proteins have been generated in mice. The result of this is often an exacerbation of the phenotypes of the single mutants (Bel et al., 1998; Akasaka et al., 2001; Kwon et al., 2003; Courel et al., 2008). We bred mice deficient for both *E2f6* and *Bmi1* in order to further define E2F6's role in polycomb-mediated repression.

Bmi1 is a core component of polycomb repressive complex one (PRC1), (Saurin et al., 2001; Levine et al., 2002) and has been shown to interact biochemically with E2F6 (Trimarchi et al., 2001). This complex maintains the repressive state of the *Hox* genes. *Bmi1* mutant mice display the same T13-L1 and L6-S1 axial skeletal transformations that *E2f6* mutant mice have (van der Lugt et al., 1994). Mice deficient for *Bmi1* also display other posterior transformations, including E, C1-C2, C7-T1, and T7-T8 (van der Lugt et al., 1994). Additionally, *Bmi1* loss compromises the proliferative capacity of primary cells. MEFs deficient for *Bmi1* exhibit an impairment in proliferation, a defect in cell cycle re-entry, and premature senescence (Core et al., 1997; Jacobs et al., 1999b; Courel et al., 2008). Loss of *Bmi1* *in vivo* results in neural and hematopoietic defects due to the

impaired proliferation and self-renewal of stem cells from these compartments (van der Lugt et al., 1994; Jacobs et al., 1999b; Molofsky et al., 2003). The impaired proliferative properties seen *in vitro* and *in vivo* can be partially attributed to the deregulation of the *Ink4a-Arf* locus (Jacobs et al., 1999b; Molofsky et al., 2005). Mice deficient for both *Bmi1* and *Ink4a-Arf* have a partial rescue of the hematopoietic and neural defects and a complete rescue of the *in vitro* defects of MEFs (Jacobs et al., 1999a; Molofsky et al., 2005).

Ink4a-Arf encodes two proteins, p16^{INK4a} and p19^{ARF}, which regulate cellular proliferation and apoptosis. *Bmi1* directly represses *Ink4a-Arf*, and *Bmi1* loss results in a derepression of this locus (Kotake et al., 2007). E2F proteins have also been shown to play a role in the regulation of *Ink4a-Arf* (Aslanian et al., 2004). Thus, to test for a genetic interaction between E2F6 and *Bmi1* in *Ink4a-Arf* and *Hox* regulation, we generated mice deficient for both of these genes.

1. E2F6 is not involved in the regulation of *Ink4a-Arf*

To investigate possible synergy between E2F6 and *Bmi1* in the regulation of the *Ink4a-Arf* locus, we did a thorough analysis of tissues and cells that affected by *Bmi1*-loss. Despite the documented role of various E2F family members in the regulation of *Arf* (Aslanian et al., 2004), we did not detect any evidence that E2F6 contributes to the regulation of the *Ink4a-Arf* locus. First, E2F6-loss had no detectable effect on the proliferation properties of MEFs, and there was no detectable derepression of p19^{ARF}. Second, we observed no further derepression of p16^{INK4a} and p19^{ARF} in *E2f6*^{-/-};*Bmi1*^{-/-} versus *Bmi1*^{-/-} MEFs and no significant difference in the proliferative or senescent properties of these two genotypes. Finally, E2F6 was not detected at the promoter of *Arf*

in ES cells. We did find that Bmi1 deficient cells have a cell cycle re-entry defect that has not been previously reported. However, the compound mutant cells do not differ significantly in their ability to re-enter the cell cycle. Previously, E2F4 had been shown to compensate for loss of E2F6 at the promoters of cell cycle genes (Zhu et al., 2004), and one hypothesis was that a lack of a genetic interaction between E2F6 and Bmi1 could be due to E2F4 or another E2F family member compensating for the loss of E2F6 at *Arf*. However, E2F6 was not found to occupy the *Arf* promoter in wildtype ES cells. This suggests that E2F6 plays little or no role in the direct regulation of *Ink4a-Arf*. Certainly, our data show that E2F6 is fully dispensable for the appropriate regulation of this locus. Consistent with this finding, the loss of E2F6 did not exacerbate the defects in either the hematopoietic compartment or the cerebellar development of the Bmi1 mutant mice that largely result from the derepression of *Ink4a-Arf*. Thus, these data suggest that E2F6 does not influence the regulation of *Ink4a-Arf* by Bmi1 by either direct or indirect mechanisms.

2. E2F6 and Bmi1 co-regulate *Hox* genes and synergize in axial skeletal development

Our analysis of *Hox* gene regulation and axial skeleton transformations clearly indicate that E2F6 and Bmi1 synergize in the regulation of the *Hox* genes. *E2f6* mutation increased the severity of *Bmi1*^{-/-} skeletal defects in a dosage-dependent manner. Deletion of only one allele of E2F6 was sufficient to increase the penetrance of C1-C2, partial C7-T1, T13-L1, and L6-S1 transformations. Further deletion of the remaining allele led to an even higher penetrance, indicating that E2F6 and Bmi1 synergistically contribute to these abnormalities. In addition to the increase in axial skeleton transformations, we have also described a novel C5 to C6 mutation where the spinous process normally present on C6

is now present on C5. This transformation was present in 10% of *E2f6*^{-/-} mice and 23% of *E2f6*^{-/-};*Bmi1*^{-/-} mice.

To determine if the increase in penetrance of the axial skeleton transformations results from further *Hox* gene deregulation, we analyzed the expression level and promoter occupancy of different *Hox* genes. Loss of *E2f6* led to further deregulation of *HoxB6* and *HoxC10*, but not *HoxA9*. To complement this analysis, we analyzed by chromatin immunoprecipitation the promoter occupancy of *Hox* genes in embryonic stem cells. Both E2F6 and Bmi1 were found at *HoxA10* and *HoxA11*, but only Bmi1 was bound at *HoxA7*. These results clearly support the conclusion that E2F6 and Bmi1 synergize in axial skeletal development.

3. Regulation of *Hox* genes and chromatin by PcG proteins

Studies of mice deficient for polycomb group proteins specifically reveal that mutation of one or two PcG proteins results in the deregulation of a subset of the thirty-nine *Hox* genes (Goebel, 1991; van der Lugt et al., 1994; Akasaka et al., 1996; Satijn et al., 1997; Schoorlemmer et al., 1997; Bel et al., 1998; Katoh-Fukui et al., 1998; del Mar Lorente et al., 2000; Akasaka et al., 2001; Storre et al., 2002; Wang et al., 2002; Courel et al., 2008). This result underscores the complexity of *Hox* gene regulation and the dynamic nature of the polycomb repressive complexes. It also disputes the accepted claim that there is a “core” PRC complex. Instead, the diverse axial skeletal phenotypes could result from the fact that there are many different variations of PRC complexes that differ slightly in their composition. These different PRC complexes could confer a different level of repression on their target genes. Studies of the roles of Bmi1 and Mel-18 complexed with Ring1 lend some support for this argument. Mel-18 has been shown

to partially compensate for Bmi1 loss in PRC1 complexes. Specifically, Mel-18 can substitute for Bmi1 in maintaining PRC1 complex integrity, but a Mel-18-Ring1 complex lacks the histone ubiquitinase activity that a Bmi1-Ring1 complex contains (Cao et al., 2005). While the Mel-18-Ring1 complex was only found in the absence of Bmi1, this result doesn't occlude the theory that this complex could exist *in vivo* at a low abundance. Therefore, this complex could potentially confer a different level of repressive activity on target genes.

Polycomb group proteins are known regulators of chromatin structure. Some PcG proteins can directly modify chromatin while others have been shown to bind directly to chromatin marks. EZH2, a component of the polycomb repressive complex 2 (PRC2), contains a SET domain and exhibits histone methyl transferase activity to histone H3 at lysine 27 (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002). This methyl mark is involved in the recruitment of PRC1. The PcG protein, polycomb (HPC in humans, M33 in mice), can bind directly to methylated lysine 27 of histone H3 (Cao et al., 2002; Muller et al., 2002). Therefore, H3K27 methylation facilitates recruitment of the polycomb protein (Pc) and mediates targeting of the PRC1 to PcG targets. Recently, it has been shown that PRC1 can ubiquitinate histone H2A. Specifically, Ring1a and Ring1b exhibit E3 ubiquitin ligase activity towards histone H2A (de Napoles et al., 2004; Wang et al., 2004; Cao et al., 2005). It was subsequently discovered that RYBP, another PcG protein, binds directly to ubiquitinated H2A *in vivo* (Arrigoni et al., 2006). It is still unknown exactly how the concerted action of H3K27 methylation and H2AK119 ubiquitination serves to repress chromatin. There is evidence to suggest the H2AK119 ubiquitination and RYBP binding serves to recruit another

histone modifier, Suv420. This histone methyltransferase was found to interact with RYBP in a yeast two-hybrid screen (JA Lees, unpublished data). Suv420 methylates histone H4 at lysine 20 (Schotta et al., 2004). Additionally, H4K20 methylation is downstream of H3K27 methylation at the inactive-X chromosome (Schotta et al., 2004). These published and unpublished results suggest a mechanism in which RYBP recruits a Suv420-containing complex to PcG targets (Figure 1). This methyl mark could lead to a very condensed state of chromatin. It is clear that a “histone code” plays a vital role in the regulation of chromatin. PcG complexes are at the core of this regulation, and their roles in regulation of transcription reach far beyond *Hox* genes.

4. E2F6 in the regulation of gliosis

Mice deficient for *E2f6* fail to undergo a proper gliotic response after neural damage (Chapter 3). Gliosis is a process in the brain that occurs in response to trauma or neuronal injury. The hallmark of gliosis is astrocyte activation, characterized by an increase in an intermediate filament protein, GFAP. There is evidence to suggest that gliosis is both neuroprotective and harmful after brain trauma. Astrocytes play a pivotal role in scavenging potentially toxic agents from the extracellular matrix (Aschner and Kimelberg, 1991; Sano et al., 1997; Marcus et al., 1998; Anderson and Swanson, 2000; Dringen, 2000; Dringen and Gutterer, 2002). After neuronal injury, this function of astrocytes lends acute protection to the surrounding neurons. However, there is increasing evidence that gliosis exacerbates the pathologies of individuals with neurodegenerative diseases. In a mouse model for Alzheimer’s disease, anti-inflammatory drugs such as ibuprofen have been shown to suppress the gliotic response and reduce amyloid plaque formation (Lim et al., 2000; Jantzen et al., 2002; Yan et al., 2003). Similarly, dispensing

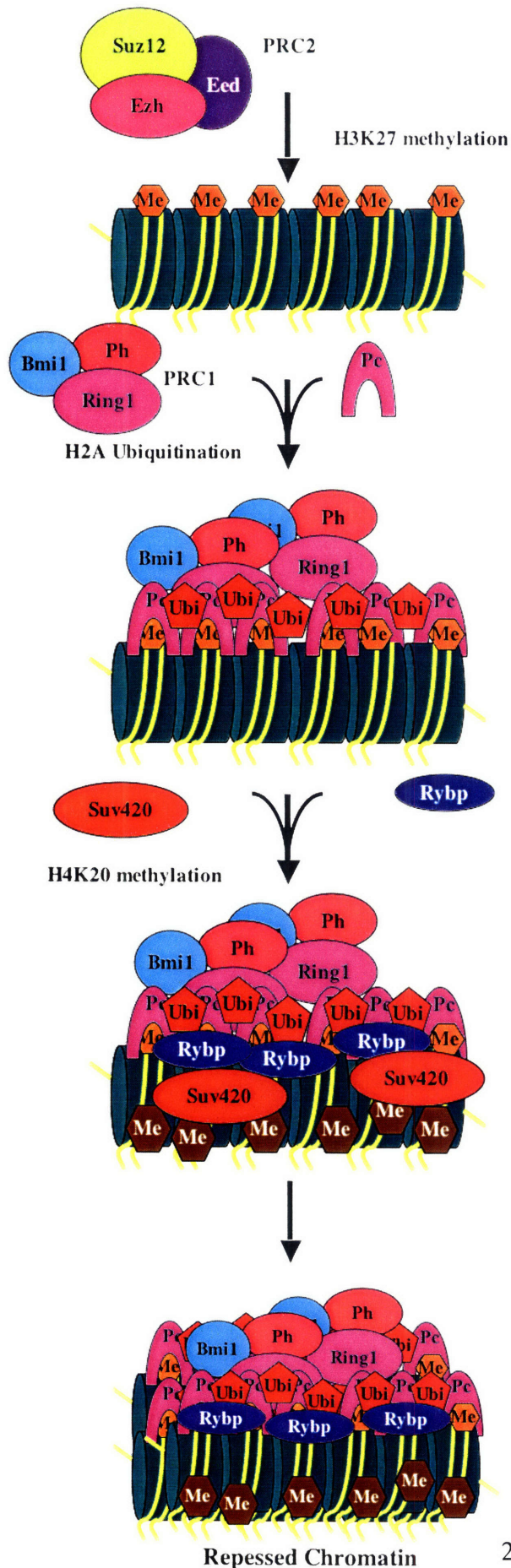


Figure 1: Hypothetical mechanism of PcG Repression of Chromatin

PcG repression is initiated by PRC2 and H3K27 methylation. This methyl mark recruits the polycomb protein and PRC1. Ring1 of PRC1 ubiquitinates histone H2A. RYBP binds directly to ubiquitinated histones. Unpublished data suggests that RYBP associates with a H4K20 methylase, Suv420. This histone methyl mark may participate in PcG repression and lead to repressed chromatin.

ibuprofen to mice or rats subjected to ischemia reduces neuronal injury and improves cerebral blood flow and neurological outcome (Kuhn et al., 1986; Cole et al., 1993; Patel et al., 1993; Antezana et al., 2003). These studies make the gliosis process a particularly interesting therapeutic target. However, little is known about the molecular mechanisms involved in triggering and sustaining a gliotic response.

There is some suggestion that neural injury induced by neurodegenerative diseases, tumorigenesis, and ischemia initiates inappropriate activation of a program required for development of the central nervous system (Liu et al., 2006). In this intriguing model, EGFR is inappropriately activated after neural damage. In the developing central nervous system, the EGFR regulates astrocyte migration and differentiation of neural precursor cells into astrocytes (Miettinen et al., 1995; Sibia and Wagner, 1995; Kornblum et al., 1998). This pathway is reactivated during neurodegeneration, ischemia, and tumorigenesis (Birecree et al., 1988; Planas et al., 1998; Jin et al., 2002; Hayashi et al., 2004). EGFR regulates genes related to reactive astrocytes and neural disorders. These genes include those involved in extracellular matrix organization, cell migration, and cytokine production (Liu and Neufeld, 2004). In response to EGFR, astrocytes produce transforming growth factor β , brain-derived neurotrophic factor, fibroblast growth factor 2, and nerve growth factor β , which may all be beneficial to the survival of neurons (Liu et al., 2006). However, activation of EGFR generates chondroitin sulfate proteoglycans and laminin, both of which interfere with axon regeneration after neural injury (McKerracher et al., 1996; Anderson et al., 1998; Koprivica et al., 2005). This interesting theory is consistent with the duality of the gliotic

response. Specifically, activation of the EGFR pathway and activation of a gliotic response present both protective and harmful attributes to surrounding neurons.

During the course of our analysis of *E2f6* mutant mice, we discovered that these mice are defective in a proper gliotic response after neural injury. Gliosis is suppressed in eight-week-old *E2f6* mutant mice with neuronal injury induced by *Bmi1* loss and five-day-old *E2f6* mutant pups injected with a neurotoxin (methylazoxymethanol - MAM). In *E2f6*^{-/-} pups injected with MAM, the phenotype was partially penetrant, with five out of seven *E2f6*^{-/-} pups showing a suppression of gliosis. Gliosis was not suppressed in five-day-old *E2f6*^{-/-} pups deficient for *Bmi1*. We hypothesize that there is a threshold level of gliotic activity that *E2f6* loss can suppress.

5. Possible molecular mechanisms of E2F6's role in gliosis

The molecular mechanism of how *E2f6* loss suppresses gliosis is unknown. To investigate this, the ideal experiment would be a microarray from *E2f6* deficient and wildtype brain extracts after neural injury. While this has not been done, other microarrays with different *E2f6*^{-/-} cell types have been performed (Oberley et al., 2003; Pohlers et al., 2005; Storre et al., 2005). In these experiments, there is one gene in particular that may provide insight into the mechanism of how *E2f6* loss suppresses gliosis. Oberley *et al.* identified *Art-27* as a gene whose mRNA transcript increases in human 293 cells transfected with a siRNA targeted to E2F6 (Oberley et al., 2003). They subsequently verified E2F6's regulation of this gene by demonstrating that E2F6 can directly bind to the promoter of *Art-27* (Oberley et al., 2003). *Art-27* is a coactivator of the androgen receptor (AR), and when *Art-27* is overexpressed, the transcriptional activity of the AR is increased (Markus et al., 2002). Interestingly, administering

gonadal hormones down-regulates reactive gliosis after a penetrating injury (Garcia-Estrada et al., 1993). Clinical and experimental studies suggest a neuroprotective role for gonadal steroids after neurological injury or disease (Jones et al., 1999; Garcia-Segura et al., 2001). The suppression of the gliotic response after administering gonadal hormones is strikingly similar to the suppression of the gliotic response in animals deficient for *E2f6*. Therefore, a likely mechanism of E2F6's requirement in the gliotic response is through the regulation of *Art-27* and androgen receptor transcriptional activity. Measuring the levels of *Art-27* in mice deficient for *E2f6* and *Bmi1* can easily test this model. Also, chromatin immunoprecipitation from the brains of wildtype and *E2f6*^{-/-} deficient mice can be used to determine if E2F6 regulates *Art-27* in neural cell types.

Gliosis plays a role in neurodegenerative disorders, and blocking the gliotic response is beneficial for mice in models of Alzheimer's disease and ischemia (Kuhn et al., 1986; Cole et al., 1993; Patel et al., 1993; Lim et al., 2000; Jantzen et al., 2002; Antezana et al., 2003; Yan et al., 2003). Therefore, an interesting experiment would be to cross mice deficient for *E2f6* to a mouse model of Alzheimer's disease. It will be important to determine whether the gliotic response is also attenuated in these mice and whether *E2f6* loss modulates the pathology of these mice. In conclusion, the gliotic response occurs in response to any neural damage. Most research done in this area suggests that this process has more harmful effects than neuroprotective effects. A better understanding of the molecular mechanisms underlying the gliotic response will likely lead to more effective therapies for neural damage.

6. Concluding Remarks

This thesis describes the role of *E2f6* in axial skeletal development and gliosis. E2F6 acts as a transcriptional repressor and can participate in polycomb mediated repression of PcG target genes. Mice deficient for *E2f6* exhibit posterior axial skeletal transformations. This phenotype reflects E2F6's role in the regulation of *Hox* genes. Additionally, these mice exhibit a suppression of gliosis after neural damage. This phenotype may be due to a deregulation of the *Art-27* gene and an activation of the androgen receptor. The *in vivo* studies presented here uncover the biological function of *E2f6* in mammalian development and propose a novel role for this gene in the regulation of neurodegeneration.

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Appendix A

E2F3a and E2F3b make overlapping but different contributions to total E2F3 activity.

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ABSTRACT

The E2F transcription factors are key downstream targets of the retinoblastoma protein tumor suppressor that control cell proliferation. E2F3 has garnered particular attention because it is amplified in various human tumors. *E2f3* mutant mice typically die around birth and *E2f3*-deficient cells have a proliferation defect that correlates with impaired E2F-target gene activation and also induction of p19^{Arf} and p53. The *E2f3* locus encodes two isoforms, E2F3a and E2F3b, which differ in their N-termini. However, it is unclear how E2F3a versus E2F3b contributes to E2F3's requirement in either proliferation or development. To address this, we use *E2f3a*- and *E2f3b*-specific knockouts. We show that inactivation of E2F3a results in a low penetrance proliferation defect *in vitro* whilst loss of E2F3b has no effect. This proliferation defect appears insufficient to disrupt normal development since *E2f3a* and *E2f3b* mutant mice are both fully viable and have no detectable defects. However, when combined with *E2f1* mutation, inactivation of *E2f3a*, but not *E2f3b*, causes significant proliferation defects *in vitro*, neonatal lethality and also a striking cartilage defect. Thus, we conclude that E2f3a and E2f3b have largely overlapping functions *in vivo* and that *E2f3a* can fully substitute for *E2f1* and *E2f3* in most murine tissues.

INTRODUCTION

The retinoblastoma protein (pRB) was the first identified tumor suppressor. pRB's tumor suppressive activity is largely dependent on its ability to regulate the E2F transcription factors. E2Fs control the cell cycle dependent transcription of genes that encode key components of the cell cycle machinery (Attwooll *et al.*, 2004; Dimova and Dyson, 2005). pRB, and its relatives p107 and p130, bind to E2fs in quiescent cells and this prevents transcription of E2F target genes *via* binding to the transactivation domain and blocking its function and by recruiting chromatin regulators which directly repress transcription (Attwooll *et al.*, 2004; Dimova and Dyson, 2005; Blais and Dynlacht, 2007). Dissociation of E2F/pocket protein complexes is triggered by mitogen-induced phosphorylation of the pocket proteins by cyclin dependent kinases, Cdks, thus allowing activation of target gene transcription by E2Fs (Trimarchi and Lees, 2002; Attwooll *et al.*, 2004; Dimova and Dyson, 2005). The majority of human tumors carry mutations that either inactivate the retinoblastoma gene or activate the Cdks that mediate pocket protein phosphorylation. This strongly suggests that the pocket-protein associated E2Fs, E2F1-5, play an important role in tumorigenesis.

The prevailing view is that individual E2F proteins are preferentially involved in either the activation or repression of E2F-responsive genes. E2F4 and E2F5 play a key role in gene repression whilst E2F1 and E2F2 play a key role in activating E2F-target genes (Trimarchi and Lees, 2002; Attwooll *et al.*, 2004; Dimova and Dyson, 2005). *E2f4* and *E2f5* are expressed constitutively but E2F1 and 2 levels increase upon cell cycle re-entry because *E2f1* and *E2f2* are E2F-responsive genes. *E2f3* is unusual in that it encodes two different proteins, E2F3a and E2F3b (He *et al.*, 2000; Leone *et al.*, 2000). These

isoforms are generated by two separate promoters that govern the expression of alternative first exons that are spliced to a common second exon (Adams *et al.*, 2000). E2F3a shares many of the properties of E2F1 and E2F2: it is inhibited by pRB in quiescent cells, recruits coactivators to E2F-responsive genes in G1 and its promoter is E2F-responsive. *E2f3b* is constitutively expressed, like *E2f4* and *E2f5*, suggesting that it could function as a transcriptional repressor (Adams *et al.*, 2000). However, in other regards, E2F3b more closely resembles the activating E2Fs. It lacks the nuclear export signals that are characteristic of the repressive E2Fs and thus remains in the nucleus after release from pocket proteins. E2F3 also associates specifically with pRB, and not p107 and p130, *in vivo*. Importantly, recent studies have linked *E2f3* amplifications to the development of human bladder, lung and prostate tumors (Feber *et al.*, 2004; Foster *et al.*, 2004; Oeggerli *et al.*, 2004; Cooper *et al.*, 2006; Oeggerli *et al.*, 2006; Hurst *et al.*, 2007).

We previously generated an *E2f3* mutant mouse strain that disrupts expression of both E2F3a and E2F3b. A large proportion of the *E2f3*-deficient mice die *in utero* or as neonates (Humbert *et al.*, 2000; Cloud *et al.*, 2002). Analysis of mouse embryonic fibroblasts (MEFs) shows that loss of *E2f3* impairs both asynchronous proliferation and mitogen-induced cell cycle re-entry (Humbert *et al.*, 2000). This latter defect correlated with two changes in gene regulation. First, there is a clear defect in the transcriptional activation of E2F-responsive genes (Humbert *et al.*, 2000). This is consistent with the notion that E2F3a, and also possibly E2F3b, contribute to gene activation. Second, E2F3-loss is sufficient to derepress the *Arf* tumor suppressor, triggering activation of p53 and expression of the cdk-inhibitor p21^{Cip1} (Aslanian *et al.*, 2004). In wildtype MEFs, the

Arf promoter is specifically occupied by E2F3b, and not other E2Fs, suggesting that E2F3b contributes to *Arf* repression *in vivo* (Aslanian *et al.*, 2004). These observations offer two distinct mechanisms by which E2F3a and/or E2F3b could promote proliferation and tumorigenesis. Given these findings, we have generated *E2f3a* or *E2f3b* mutant mouse strains to determine how each of these isoforms contributes to E2F3's key roles in cellular proliferation and normal development.

RESULTS

E2F3b binding to the *Arf* promoter is not required for *Arf* repression.

To create *E2f3a*- and *E2f3b*-specific mutants we used recombineering to replace the relevant ATG translation start codon with a single *loxP* sequence (Figure 1A and Supplementary Information). ES cell lines shown to be correctly targeted by Southern blotting (Figure 1b) were used to produce *E2f3a* and *E2f3b* mutant mouse strains in both mixed (C57BL/6 x 129S/v) and pure 129S/v genetic backgrounds. Our first goal was to verify the specificity of our mutation strategy. To this end, we generated MEFs from mutant and wildtype littermates and conducted western blotting using a pan-E2F3 antibody, LLF3#2G2, which recognizes a C-terminal sequence common to both E2F3 isoforms (Parisi *et al.*, 2007). This analysis confirmed that the introduced mutations specifically abolished the expression of either E2F3a or E2F3b (Figure 1c). Moreover, there was no detectable change in the levels of the remaining E2F3 isoform (Figure 1c) or in the levels of E2F1 or E2F4 (data not shown).

Figure 1

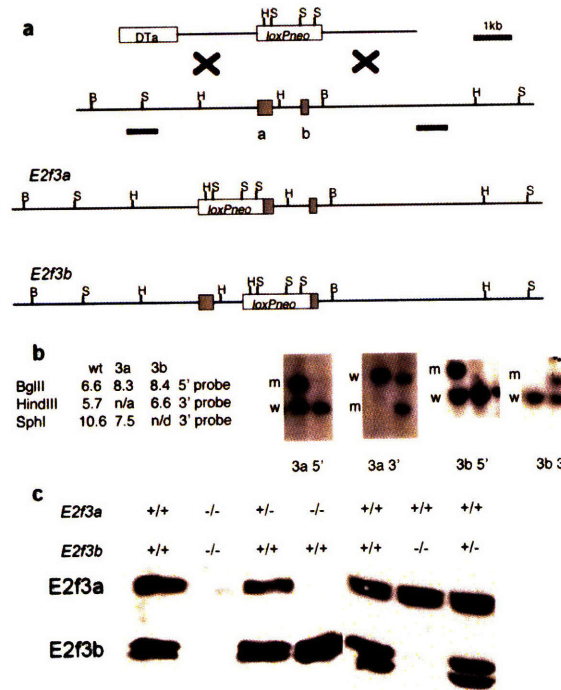


Figure 1: Mutation of *E2f3a* or *E2f3b* by gene targeting.

(a) Schematic of the targeting construct and the endogenous *E2f3* locus. The *E2f3a* and *E2f3b* exons are represented by the shaded boxes. The common second exon is 65kb from these exons. DTa represents the diphtheria toxin negative selection cassette and *loxPneo* the *loxP* site flanked PGKEM7neobpA positive selection cassette. The two loci after the predicted homologous recombination event are shown for *E2f3a* and *E2f3b*. The two lines underneath the *E2f3* locus represent the 5' and 3' probe sequences used for Southern analysis of the homologous recombination event. (b) The predicted sizes of the genomic DNA fragments identified by these probes are shown along with Southern analysis of representative targeted clones. In each case one targeted clone and one non-targeted clone is shown. (c) Western blotting analysis of protein extracts derived from mouse embryo fibroblasts (MEFs) of the indicated genotypes. In wildtype MEFs, both E2F3a and E2F3b are detected. Both of these isoforms run as doublets. In *E2f3* mutant MEFs both isoforms are absent. Mutation of *E2f3a* or *E2f3b* leads to specific loss of only the mutated isoform indicating that the targeting strategy successfully disrupted expression of each isoform separately.

Our previous studies showed that E2F3 mutation lead to the induction of *Arf* in MEFs, and implicated the E2F3b isoform as a direct transcriptional repressor of *Arf* (Aslanian *et al.*, 2004). Thus, we wanted to establish how the loss of either E2F3a or E2F3b affects the regulation of *Arf*. First, we performed chromatin immunoprecipitation (ChIP) experiments to examine E2F binding to the *Arf* promoter in MEFs derived from *E2f3a*^{-/-} or *E2f3b*^{-/-} embryos alongside their wildtype littermate controls (Figure 2). We used a pan-E2F3 antibody for these studies to allow direct comparison of the binding of E2F3a versus E2F3b. Consistent with our previous studies, we found that *Arf* is specifically bound by E2F3 in wildtype MEFs. This is in clear contrast to a classic E2F-responsive gene, *p107*, which shows significant occupancy by both E2F3 and E2F4. As expected, mutation of *E2f3a* did not alter the anti-E2F3 ChIP signal detected at *Arf* (Figure 2a). This supported our prior conclusion that E2F3b is the major E2F bound to *Arf* in MEFs but it does not preclude the possibility that both E2F3a and E2F3b can occupy the *Arf* promoter in wildtype MEFs. In the absence of E2F3b, we now observed ChIP signals with antibodies to E2F3, E2F4 and, to a lesser extent E2F1, at *Arf* (Figure 2b). Similar results were observed in three other isoform specific mutant lines analyzed (data not shown). Thus, we conclude that E2F3a, and also other E2Fs, are able to bind to the *Arf* promoter in place of E2F3b in *E2f3b*^{-/-} MEFs.

To determine whether the loss of E2F3b or E2F3a increases the levels of p19^{Arf} and p21^{Cip1} as observed in *E2f3* and *E2f1;E2f2;E2f3* mutant MEFs (Wu *et al.*, 2001; Aslanian *et al.*, 2004; Sharma *et al.*, 2006; Timmers *et al.*, 2007), we analyzed the levels of these proteins in serum starved mutant and wildtype MEFs generated from littermate embryos. We conducted these studies using both mixed (C57BL/6 x 129Sv) and pure

129Sv background MEFs, since the *E2f3* mutant phenotype is always stronger in the pure 129Sv background (Cloud *et al.*, 2002). Irrespective of the genetic background, the levels of p19^{Arf} or p21^{Cip1} were not elevated in the *E2f3b* mutant cells (n=8 independent mutant cell lines) relative to wildtype littermate controls (Figure 2d). The mixed (C57BL/6 x 129Sv) background *E2f3a*^{-/-} MEFs did not display any detectable defect in this, or any other, assay (data not shown). In contrast, the pure 129Sv background *E2f3a*^{-/-} MEFs showed somewhat variable phenotypes. Five of the six lines tested showed either no upregulation of either p19^{Arf} or p21^{Cip1}, or they had slightly elevated levels of just one of these proteins (Figure 2c, data not shown). The remaining line had increased levels of both *Arf* and *p21* (Supplementary Figure 1). These changes are subtle. However, the E2F3a-deficient MEFs seem to be more predisposed to upregulate p19^{Arf} and/or p21^{Cip1} than wildtype controls. Taken together, these data show that the robust activation of the p19^{Arf}-p53-p21^{Cip1} network that occurs in *E2f3* mutant MEFs cannot be reproduced by the loss of either E2F3b or E2F3a. Since E2F3b is the predominant *Arf* promoter binder in wildtype cells but E2F3a takes its place in *E2f3b*-deficient MEFs and *Arf* and *p21* regulation is subtly impaired in *E2f3a*-deficient MEFs, we conclude that E2F3a and E2F3b can play overlapping roles in the appropriate regulation of the p19^{Arf}-p53-p21^{Cip1} network.

E2F3a and E2F3b play overlapping roles in controlling asynchronous proliferation and cell cycle entry.

E2f3-loss impairs the proliferation properties of MEFs and this correlates with both the derepression of *Arf* and the failure to appropriately induce classic E2F-

Figure 2

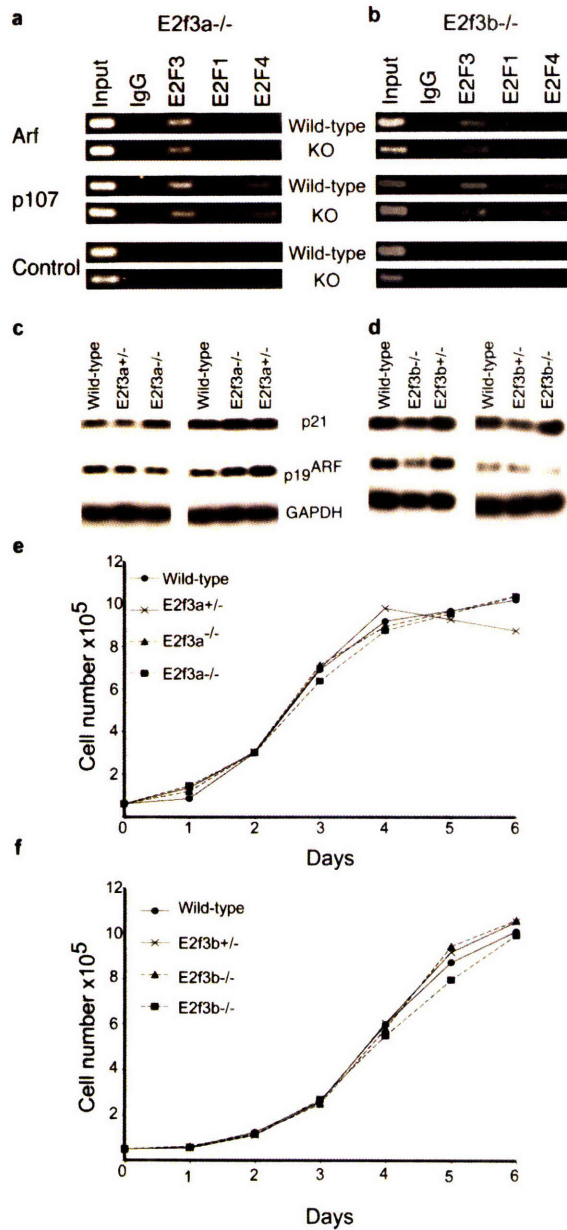


Figure 2: *Arf* promoter regulation, p19^{Arf} and p21^{Cip1} expression and asynchronous proliferation properties of *E2f3a*⁻ and *E2f3b*⁻ deficient MEFs. (a) Chromatin immunoprecipitation (ChIP) was performed using asynchronously proliferating wildtype and *E2f3a*⁻ littermate MEFs, or (b) wildtype and *E2f3b*⁻ littermate MEFs. Sonicated cross-linked chromatin was immunoprecipitated with antibodies to E2F3, E2F1, E2F4, or control IgG. The purified DNA was analyzed by PCR with primers specific for the *p107* or *Arf* promoters, or a control sequence lacking E2F binding sites (1kb upstream of a control promoter). Input, 0.5% of chromatin in IP reaction was analyzed by PCR. These analyses show that in the absence of E2F3b, E2F3a is detected bound to the *Arf* promoter. (c) The majority of *E2f3a* mutant MEFs show little or no increase in p19^{Arf} and p21^{Cip1} levels relative to wildtype controls, as illustrated by western blot analysis of two representative sets of serum arrested MEFs. GAPDH is shown as a loading control. (d) No increase in p19^{Arf} or p21^{Cip1} levels are observed in *E2f3b* mutant MEFs relative to wildtype littermate controls. (e) *E2f3a*⁻, *E2f3a*^{+/-} and *E2f3a*^{+/+} MEFs or (f) *E2f3b*⁻, *E2f3b*^{+/-}, and *E2f3b*^{+/+} MEFs were assayed for asynchronous proliferation.

Cells were plated in duplicate at 6x10⁴/3cm dish and their growth monitored by daily counting for six days. No significant growth defect was observed in the isoform specific mutant cells.

responsive genes (Humbert *et al.*, 2000; Aslanian *et al.*, 2004). Having shown that loss of E2F3a or E2F3b results in occasional or no increase in p19^{Arf} and p21^{Cip1} levels respectively, we wished to assess whether cell proliferation was compromised. To test this we compared the properties of mutant versus wildtype MEFs in standard proliferation assays, and also assayed their ability to re-enter the cell cycle from a serum starvation-induced quiescent state. The vast majority of the *E2f3b* mutant MEF lines had proliferation properties that were indistinguishable from the controls irrespective of whether they were generated in the mixed (6/7 lines tested) or pure 129Sv (6/7 lines tested) genetic backgrounds (Figure 2f shows representative lines). Moreover, none of the three of *E2f3b* mutant lines tested in cell cycle re-entry experiments showed any detectable defect in the kinetics of cell cycle progression, as judged by analysis of BrdU incorporation (Figure 3a shows a representative line). In tandem with this analysis, we also assessed mRNA expression levels of three E2F target genes, *Cyclin E*, *Cyclin A* and *p107* as well as *Arf* and *p21^{Cip1}*. Consistent with lack of proliferation and re-entry defects, we saw no significant difference in levels of these mRNAs in control versus *E2f3b*^{-/-} MEFs using two independent sets of lines (Figure 3b and data not shown). Thus, we conclude that E2F3b-loss has no dramatic affect on either *Arf* levels or cell cycle regulation.

As with our analysis of *Arf* regulation, the properties of the *E2f3a*^{-/-} MEFs were more variable. The majority (4/6) of the pure background *E2f3a*^{-/-} lines showed no detectable defect in asynchronous proliferation (Figure 2e shows two representative lines). Accordingly, their kinetics of cell cycle re-entry and regulation of *Cyclin E*, *Cyclin A*, *p107*, *Arf* and *p21* mRNAs was similar to that of wildtype littermate controls (Figure

3; data not shown). In contrast, two of the six *E2f3a*^{-/-} lines grew more slowly than the wildtype littermate lines in asynchronous proliferation assays (Supplementary Figure 1). They also had a significant defect in cell cycle re-entry and this was associated with a failure to appropriately induce the transcription of classic E2F-responsive targets (Supplementary Figure 1). Importantly, one of these cell lines had an associated upregulation of *Arf* and *p21* but the other showed little change in these mRNAs (Supplementary Figure 1). Given these findings, we conclude that *E2f3a*^{-/-} MEFs have a partially penetrant phenotype that can affect both the p19^{Arf}-p53-p21^{Cip1} network and cell cycle regulation. In one *E2f3a*^{-/-} cell line, the defects in E2F target induction and cell cycle progression appeared to be independent of changes in *Arf* and *p21* expression, suggesting that these events can be uncoupled. Finally, our data show that the loss of either E2F3a or E2F3b has far less impact on cell cycle regulation than the combined loss of both E2F3 isoforms (Humbert *et al.*, 2000), indicating that these two proteins, or other members of the E2F family, can largely substitute for one another in the regulation of cell cycle genes.

Neither *E2f3a* nor *E2f3b* are required for viability

To assess the role of E2F3a in normal development, *E2f3a*^{+/-} animals were intercrossed in either a mixed (C57BL/6x129Sv) or 129Sv background. A similar strategy was used to test the consequences of mutating the *E2f3b* allele. In each case the frequency of mutant animals was not statistically significantly different from the expected Mendelian frequency (Table 1 and 2). This is in contrast with the mutation of *E2f3* (both a+b isoforms) which results in reduced viability in a mixed background and

Figure 3

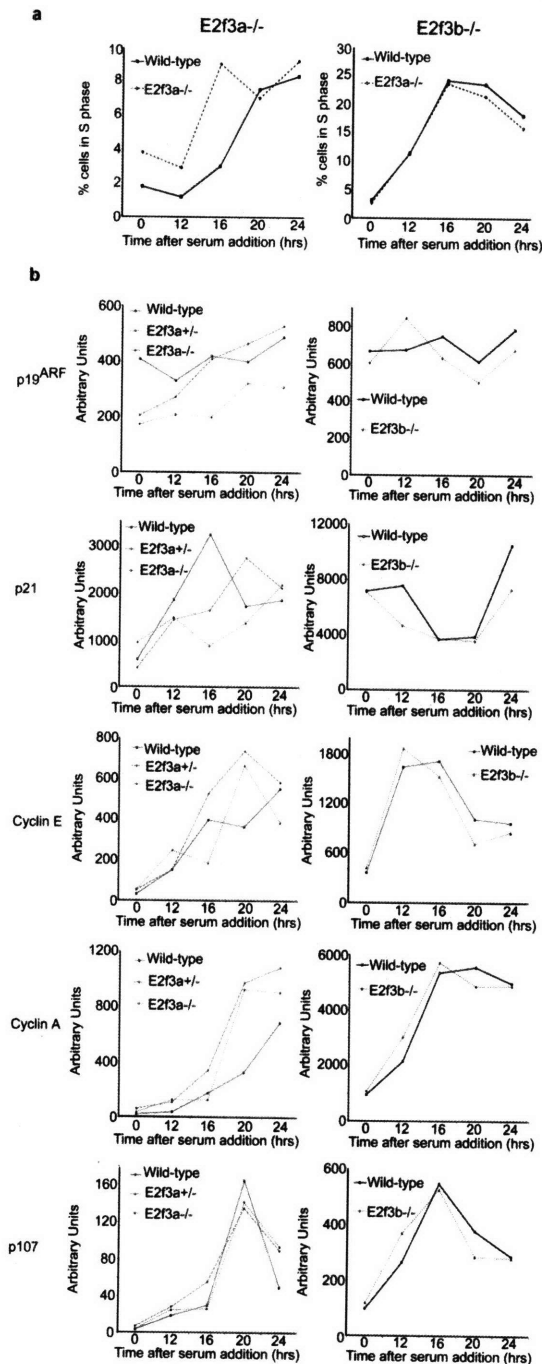


Figure 3: Cell cycle re-entry properties of *E2f3a*^{-/-} and *E2f3b*^{-/-} deficient MEFs.

E2f3a^{-/-} and *E2f3b*^{-/-} MEFs were serum starved (T₀) and induced to enter into the cell cycle with serum alongside wildtype littermate controls. (a) Entry into the cell cycle was analyzed by BrdU incorporation at the indicated times (hours) followed by propidium iodide staining and FACS analysis, the percentage of cells in S-phase (BrdU labeled) is plotted. (b) Quantitative PCR analyses of the mRNA levels of *Arf* and *p21* as well as the E2F target genes *Cyclin E*, *Cyclin A* and *p107* during the cell cycle re-entry experiment.

embryonic lethality in a 129Sv background (Humbert *et al.*, 2000; Cloud *et al.*, 2002). In addition, histological analyses of *E2f3a*^{-/-} adults and *E2f3b*^{-/-} adults (n=8 mutant animals of each isoform, 4 of each sex) in comparison with wildtype littermates on either background did not identify any distinct pathology. Additionally, no distinct phenotype associated with either E2F3 isoform mutation has been observed in aging colonies (data not shown).

Table 1. Mutation of *E2f3a* does not cause a significant reduction in viability

Background	<i>E2f3a</i> ^{-/-} observed	χ^2 test p value
<i>E2f3a</i> Mix (C57BL/6 x 129Sv)	24% (23/94)	0.534
<i>E2f3a</i> Pure (129Sv)	23% (60/257)	0.615

E2f3a^{+/-} mice on the indicated backgrounds were crossed and the pups genotyped at three weeks of age. The indicated frequency of mutant animals was not significantly different from the expected frequency as judged by a χ^2 test.

Table 2. Mutation of *E2f3b* does not cause a significant reduction in viability

Background	<i>E2f3b</i> ^{-/-} observed	χ^2 test p value
<i>E2f3b</i> Mix (C57BL/6 x 129Sv)	27% (47/176)	0.248
<i>E2f3b</i> Pure (129Sv)	33% (28/85)	0.256

E2f3b^{+/-} mice on the indicated backgrounds were crossed and the pups genotyped at three weeks of age. The observed frequency of mutant animals was not significantly different from the expected frequency as judged by a χ^2 test.

E2f3a* but not *E2f3b* is required for viability and proliferation control in the absence of *E2f1

We have previously shown that the additional mutation of one or both *E2f1* alleles causes *E2f3* mutant embryos to die at progressively earlier timepoints, indicating

significant functional overlap between E2F1 and E2F3 (Cloud *et al.*, 2002). Thus, to further test the relative roles of the two E2F3 isoforms, we intercrossed the *E2f3a* and *E2f3b* mutants with *E2f1* mutant mice to determine the phenotypes of compound mutant mice in a mixed (C57BL/6x129Sv) background. Remarkably, we found that *E2f1*^{-/-}; *E2f3b*^{-/-} animals were present at the expected Mendelian frequency when weaned at three weeks of age (Table 3). Moreover, these mice were viable and fertile, and histological analyses of *E2f1*^{-/-}; *E2f3b*^{-/-} (n=5) versus *E2f1*^{+/-}; *E2f3b*^{+/-} (n=3) littermate controls showed that the compound mutants did not display any defects beyond those previously reported in the *E2f1* mutant animals (data not shown). Thus, *E2f3b*-loss does not exacerbate the phenotypic consequences of *E2f1*-deficiency. In contrast, *E2f3a* mutation had a dramatic effect. First, the *E2f1*^{-/-}; *E2f3a*^{-/-} mice were greatly under-represented (p=0.021) at three weeks of age (Table 4). Moreover, the two surviving double mutants weighed less than one-third of their *E2f1*^{-/-}; *E2f3a*^{+/-} littermates (data not shown). To determine the time of death, we conducted *E2f1*^{-/-}; *E2f3a*^{+/-} intercrosses and recovered the pups at P1 for genotyping and also histological analysis. At P1, the observed number of living *E2f1*^{-/-}; *E2f3a*^{-/-} pups was not statistically different from the expected number (Table 4). This, along with daily monitoring of other litters, showed that the *E2f1*; *E2f3a* compound mutants die as neonates.

The histological analyses of P1 *E2f1*^{-/-}; *E2f3a*^{-/-} pups (n=6) and paired littermate controls did not identify any tissue specific defects that could obviously account for the death of the *E2f1*^{-/-}; *E2f3a*^{-/-} neonates (data not shown). Instead, we believe that this results from their failure to thrive because of their small size. Histological analysis did, however, reveal a striking defect in cartilage morphology in the *E2f1*; *E2f3a* compound mutants.

Table 3. E2f1;E2f3b double mutant animals arise at the expected frequency

Cross conducted	<i>E2f1</i> ^{-/-} ; <i>E2f3b</i> ^{-/-} observed	Expected
<i>E2f1</i> ^{+/-} ; <i>E2f3b</i> ^{+/-} x <i>E2f1</i> ^{-/-} ; <i>E2f3b</i> ^{+/-}	25% (6/25)	25%
<i>E2f1</i> ^{-/-} ; <i>E2f3b</i> ^{+/-} x <i>E2f1</i> ^{+/-} ; <i>E2f3b</i> ^{+/-}	12.5% (2/16)	12.5%
<i>E2f1</i> ^{+/-} ; <i>E2f3b</i> ^{-/-} x <i>E2f1</i> ^{+/-} ; <i>E2f3b</i> ^{+/-}	16.7% (3/18)	12.5%
<i>E2f1</i> ^{-/-} ; <i>E2f3b</i> ^{-/-} x <i>E2f1</i> ^{+/-} ; <i>E2f3b</i> ^{+/-}	23% (3/13)	25%
<i>E2f1</i> ^{-/-} ; <i>E2f3b</i> ^{-/-} x <i>E2f1</i> ^{+/-} ; <i>E2f3b</i> ^{-/-}	40% (2/5)	50%
χ^2 test p value (sum)		0.98

The indicated crosses were performed using mice on a C57BL/6 x 129Sv background and the pups genotyped at three weeks of age. The frequency of double mutant animals is shown and was determined not to be statistically significantly different from the expected frequency using a χ^2 test.

Table 4. Mutation of E2f1 and E2f3a significantly reduces viability

Age	<i>E2f1</i> ^{-/-} ; <i>E2f3a</i> ^{+/-}	<i>E2f1</i> ^{-/-} ; <i>E2f3a</i> ^{+/-}	<i>E2f1</i> ^{-/-} ; <i>E2f3a</i> ^{-/-}	χ^2 test p value
1 Day old	16	20	9	0.615
3 Weeks old	51	89	2	0.021

Mixed (C57BL/6 x 129Sv) background E2f1^{-/-};E2f3a^{+/-} mice were intercrossed and the number of pups of each genotype was determined at the indicated ages. The observed frequency of *E2f1*^{-/-};*E2f3a*^{-/-} mice was not significantly different from expected at birth but significantly lower than expected at three weeks of age as determined by χ^2 test.

This was observed in various bones, including the spine, but was most apparent in the long bones (Figure 4; data not shown). Thus, we analyzed the defect in this setting. The *E2f1*^{-/-};*E2f3a*^{-/-} femurs stained appropriately with Alcian Blue, indicating that mature cartilage was formed (data not shown), but the constituent chondrocytes were disorganized and/or displayed abnormal morphologies (Figure 4a). First, chondrocytes within the columnar layer of *E2f1*;*E2f3a* compound mutant epiphyseal plates did not form stacked columns typical of chondrocytes in this region, and many of the *E2f1*^{-/-}

;E2f3a^{-/-} cells lack the condensed “bean shape” cytoplasm that is characteristic of their wildtype counterparts but instead had a more diffuse cytoplasm resembling pre-hypertrophic chondrocytes (Figure 4a). Second, chondrocytes within the resting, columnar and hypertrophic zones of the *E2f1;E2f3a* compound mutant femurs appear considerably larger than those in control *E2f1^{-/-};E2f3a^{+/+}* embryos (Figure 4a). To further investigate this defect, we measured chondrocyte sizes in comparable zones of *E2f1^{-/-};E2f3a^{-/-}* versus littermate control *E2f1^{-/-};E2f3a^{+/+}* femurs from two different P1 litters (Figure 4b). In each of the three zones we detected two clear differences between the two genotypes; the *E2f1^{-/-};E2f3a^{-/-}* chondrocytes showed a much greater range of cell sizes than their *E2f1^{-/-}* littermate controls and they displayed a statistically significant 1.5-2.0 fold increase in their mean surface area ($p < 0.001$ or < 0.0001). Analyses of 18.5dpc embryos gave identical results (Fig 4C, Supplementary Figure 2). Age-matched *E2f3a* mutant embryos showed a negligible increase in cell size (1.08 fold) only in the hypertrophic chondrocytes whilst *E2f1* mutant embryos showed a modest 1.2-1.4 fold increase in cell size in all of the chondrocyte zones (Fig 4C, Supplementary Figure 2). These results show that *E2f1* mutation causes a subtle chondrocyte defect and this is exacerbated by the mutation of *E2f3a*. Collagen II and collagen X, markers of chondrocytes and hypertrophic chondrocytes respectively, were both expressed in the appropriate regions of the mutant embryos (Supplementary Figure 3). Thus, the increase in chondrocyte size following loss of E2F activity does not appear to result from premature activation of the hypertrophic program.

Figure 4

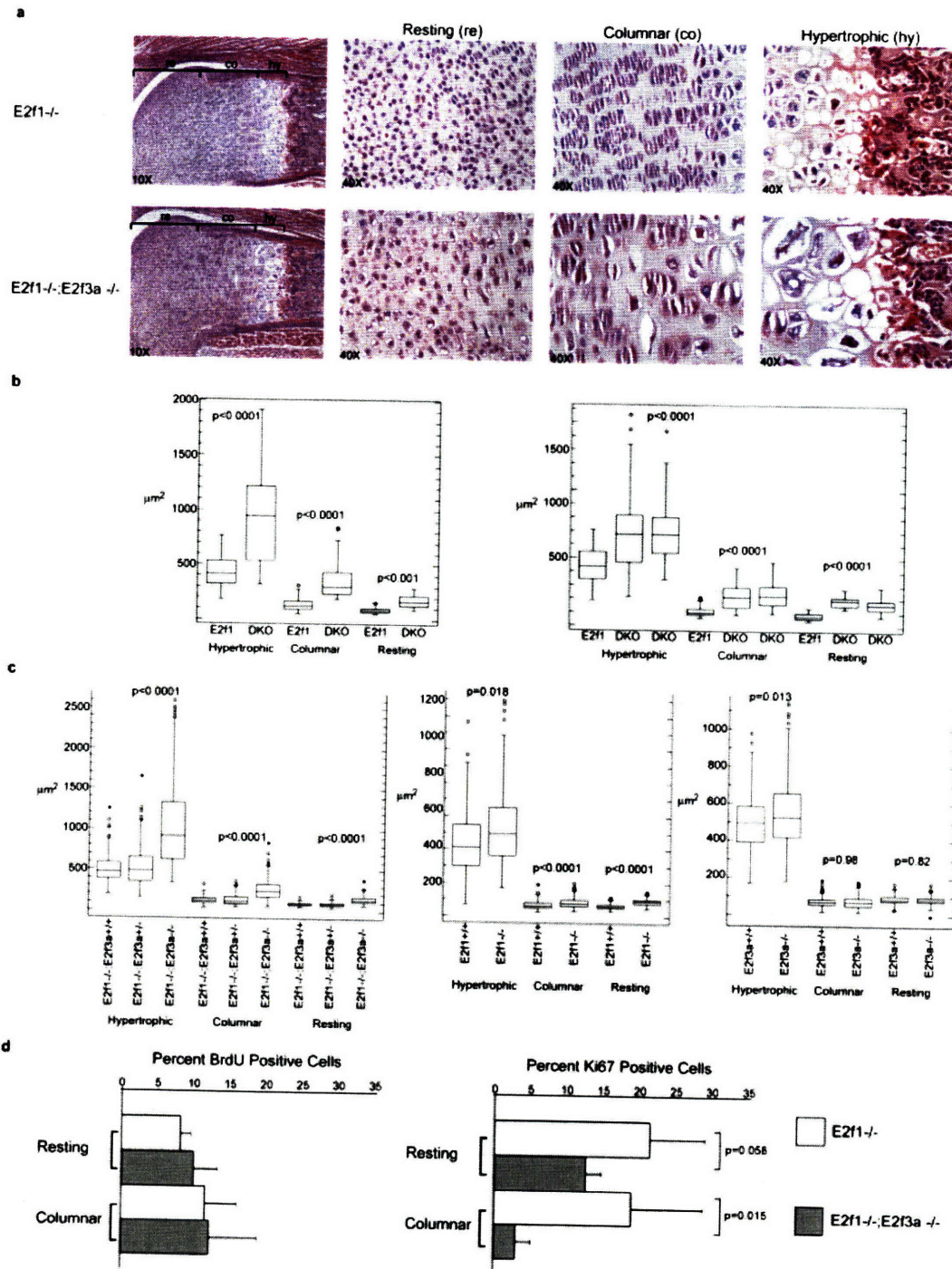


Figure 4: Mutation of *E2f1* and *E2f3a* results in abnormal cartilage morphology.

(a) Hematoxylin and eosin stained sections of hind leg femoral epiphyses from *E2f1*^{-/-} and *E2f1*^{-/-};*E2f3a*^{-/-} mutant littermate P1 pups. The zones of resting, columnar and prehypertrophic/hypertrophic chondrocytes are shown. Cells in all three zones are larger in *E2f1*;*E2f3a* mutants in comparison with control *E2f1* mutants, in addition cells within the columnar zone are disorganized in the *E2f1*;*E2f3a* mutants in comparison with control *E2f1* mutants and don't form the typical stacked columns. (b) From photographs of *E2f1* mutant and littermate *E2f1*;*E2f3a* mutant femurs chondrocyte sizes in each zone were measured and the data displayed as box plots for two representative P1 litters illustrating the universal increase in cell size. The data was also analyzed using a student's t-Test and, in all zones, cells in the *E2f1*;*E2f3a* mutants were statistically significantly larger than those in the same zones of the *E2f1* mutants (p values indicated). (c) Box plot analyses of chondrocyte size quantification from three or more *E2f1*;*E2f3a* mutant embryos and littermate controls of the indicated genotypes at 18.5dpc show a similar phenotype (left panel). A weaker but statistically significant increase in cell size is observed in 18.5dpc *E2f1*^{-/-} embryos relative to wildtype controls (middle panel). A minor cell size increase is also seen in 18.5dpc *E2f3a*^{-/-} embryos versus wildtype controls only in the hypertrophic chondrocytes (right panel), p values derived from Student's t-test analysis of the mean cell sizes are shown. (d) Quantification of proliferation markers in resting and columnar chondrocytes at 18.5 dpc. Immunohistochemistry was used to label cells that had incorporated BrdU or expressed Ki67. No significant difference in BrdU labeling was detected but Ki67 was detected in a smaller percentage of cells within the columnar layer in *E2f1*;*E2f3a* mutants relative to *E2f1* mutants (mean +/- s.d. and p values indicated).

E2F3 and E2F1 have both been linked to the regulation of proliferation and cell death. Thus, we examined the state of both processes by screening matched femoral sections by immunohistochemical staining for Ki67, a proliferation marker and known E2F-responsive gene, and incorporated BrdU, to detect replicating cells, and TUNEL or cleaved caspase 3 staining to detect apoptotic cells. Given the mild chondrocyte defect in the *E2f1*^{-/-} mice, we first compared femurs from *E2f1*^{-/-} embryos at 18.5dpc with those of wildtype littermate controls. In these two genotypes, we saw no statistically significant difference in BrdU or Ki67 labeling of the cells in the resting and columnar zones (data not shown). We could not assess cells in the hypertrophic zone, since these typically lose their nuclei. We next compared femurs from *E2f1*^{-/-};*E2f3a*^{-/-} versus *E2f1*^{-/-};*E2f3a*^{+/+} littermates at 18.5dpc. There was no detectable difference in the level of apoptosis between these two genotypes (data not shown). Similarly, there was no statistically significant difference in the percentage of BrdU-positive cells in either the resting or columnar zones of *E2f1*^{-/-};*E2f3a*^{-/-} versus *E2f1*^{-/-};*E2f3a*^{+/+} femurs at 18.5dpc (Figure 4d). In contrast, analysis of adjacent sections showed the percentage of Ki67 labeled cells was modestly decreased in the resting zone (p=0.058), and significantly decreased in the columnar zone (p=0.015), of the *E2f1*^{-/-};*E2f3a*^{-/-} versus *E2f1*^{-/-};*E2f3a*^{+/+} embryos (Figure 4d). We therefore conclude that the combined loss of E2F1 and E2F3a impairs the expression of at least one E2F-target gene, Ki67, in chondrocytes. Although there is no detectable impairment of DNA replication at this timepoint, these data suggest that loss of E2F1 and E2F3a somehow impairs cell cycle progression.

To further explore this possibility, we generated MEFs from *E2f1*^{-/-};*E2f3a*^{-/-} embryos and compared their properties to *E2f1*^{-/-} littermate controls, since it is well

Figure 5

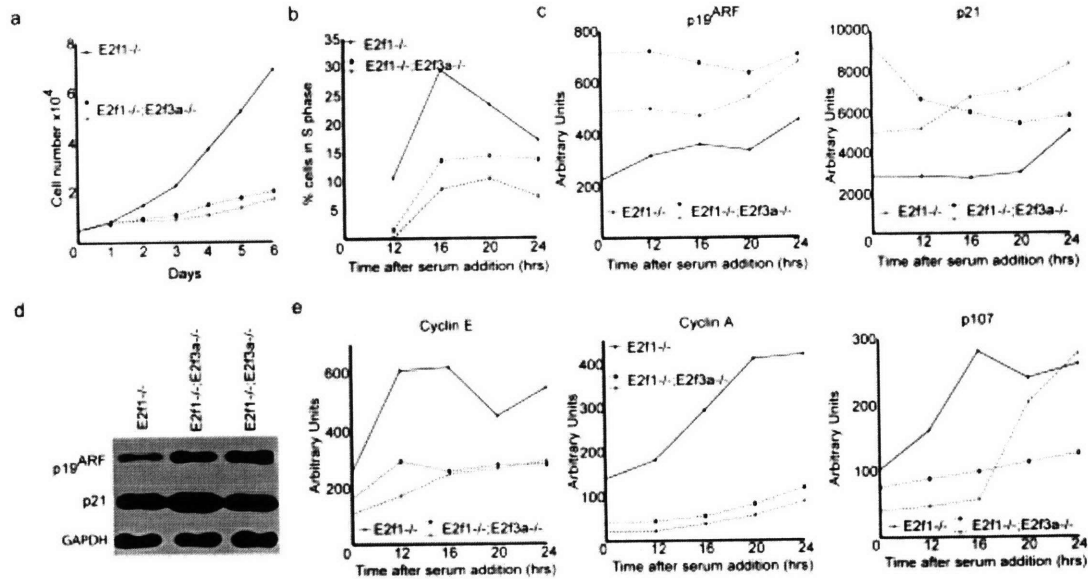


Figure 5: *E2f1*;*E2f3a* MEF lines show defects in proliferation, cell cycle re-entry and E2F target gene induction.

(a) *E2f1*^{-/-};*E2f3a*^{-/-} MEFs were assayed for asynchronous proliferation alongside *E2f1*^{-/-} littermate controls. Cells were plated in duplicate at 6×10^4 /3cm dish and their growth monitored by daily counting for six days. (b) *E2f1*^{-/-} and *E2f1*^{-/-};*E2f3a*^{-/-} littermate MEFs were serum starved (T_0) and induced to enter into the cell cycle with serum. Entry into the cell cycle was analyzed by BrdU incorporation at the indicated times (hours) followed by propidium iodide staining and FACS analysis, the percentage of cells in S-phase (BrdU labeled) is plotted. (c) Quantitative PCR analyses of the mRNA expression levels of *Arf* and *p21* during cell cycle re-entry. (d) Western blotting for *p19^{Arf}*, *p21^{Cip1}* and *GAPDH* (as a loading control) in serum starved MEFs. (e) Quantitative PCR analyses of the mRNA expression levels of the E2F target genes *Cyclin E*, *Cyclin A* and *p107* in *E2f1*^{-/-} and *E2f1*^{-/-};*E2f3a*^{-/-} littermate MEFs during the cell cycle re-entry experiment. Both *E2f1*^{-/-};*E2f3a*^{-/-} MEF lines showed reduced proliferation, reduced S-phase entry, increased *p19^{Arf}* and *p21^{Cip1}* expression and lower levels of E2F target gene expression.

established that E2F1-deficiency does not significantly impair MEFs (Field *et al.*, 1996; Humbert *et al.*, 2000). Three of the four *E2f1^{-/-};E2f3a^{-/-}* MEF lines analyzed were impaired in both asynchronous proliferation and cell cycle re-entry (Figure 5a,b; data not shown). Accordingly, we observed an upregulation in both *Arf* and *p21* mRNA and protein levels (Figures 5c,d), and poor induction of the classic E2F-responsive genes *Cyclin E*, *Cyclin A* and *p107* following cell cycle entry (Figure 5e). This spectrum of defects is strikingly similar to that seen in *E2f3^{-/-}* MEFs, lacking both E2F3a and E2F3b (Humbert *et al.*, 2000; Aslanian *et al.*, 2004). Consistent with the viability of *E2f1^{-/-};E2f3b^{-/-}* mice, preliminary studies indicate that the *E2f1^{-/-};E2f3b^{-/-}* MEF lines don't have a proliferation defect (data not shown). Taken together, these data suggest that there is significant overlap in the functions of E2F1, E2F3a and E2F3b. Based on the relative phenotypes of both the single mutant MEFs and the compound mutant mice, we conclude that, of the two isoforms, E2F3a seems to play the more important role in both cell cycle control and normal development.

DISCUSSION

The E2F transcription factors are key downstream targets of the pRB tumor suppressor. Considerable attention has focused on E2F3 because it is amplified in a variety of human tumors including bladder, lung and prostate tumors (Feber *et al.*, 2004; Foster *et al.*, 2004; Oeggerli *et al.*, 2004; Cooper *et al.*, 2006; Oeggerli *et al.*, 2006; Orlic *et al.*, 2006; Hurst *et al.*, 2007). We, and others, have previously generated *E2f3* mutant mouse strains that eliminate expression of both E2F3a and E2F3b (Humbert *et al.*, 2000; Wu *et al.*, 2001). Analyses of these models show that E2F3 is of central importance.

First, E2F3 promotes the development of various tumor types (Ziebold *et al.*, 2003; Parisi *et al.*, 2007). Second, it is the only *E2f* knockout that leads to embryonic lethality or to a profound defect in cellular proliferation (Humbert *et al.*, 2000; Wu *et al.*, 2001; Cloud *et al.*, 2002). Notably, this proliferation defect correlates with two distinct changes: there is an impaired activation of E2F responsive genes and also a derepression of *Arf* and a consequent activation of the p53-p21^{Cip1} anti-proliferative response (Aslanian *et al.*, 2004). Our prior analysis strongly suggested that this latter defect reflects a specific role for E2F3b in the transcriptional repression of *Arf* (Aslanian *et al.*, 2004). However, it was unclear to what extent E2F3a versus E2F3b contributes to either the cellular or developmental requirements for *E2f3*. In this study, we address this question through the generation and analyses of *E2f3a* and *E2f3b*-specific mutant mouse strains. These analyses show that the two isoforms have largely overlapping functions. First, the loss of E2F3a has a low penetrance effect on the asynchronous proliferation and cell cycle re-entry properties of MEFs. In contrast, we found that E2F3b is not required for the appropriate repression of *Arf*, asynchronous proliferation, or cell cycle re-entry in MEFs. Second, in stark contrast to the high frequency of late stage embryonic or early neonatal lethality that results from *E2f3*-inactivation, we find that the *E2f3a*^{-/-} and *E2f3b*^{-/-} mice are born at the expected frequency and they live into adulthood without any significant pathology. Thus, these data show that the presence of either E2F3a or E2F3b is largely sufficient to fulfill the essential role of the *E2f3* locus in both the control of cellular proliferation and normal development.

We have previously demonstrated a strong synergy between *E2f3* and *E2f1* by showing that the combined mutation of these genes causes lethality between day 10 and

12 of gestation (Cloud *et al.*, 2002). Given this observation, we further probed the relative roles of E2F3a and E2F3b by intercrossing the isoform-specific knockouts with *E2f1* mutant mice. Remarkably, we found that the *E2f1^{-/-};E2f3b^{-/-}* double mutant mice were fully viable and had only the limited spectrum of developmental defects that are characteristic of the *E2f1*-deficient animals. Since *E2f1^{-/-};E2f3^{-/-}* embryos die in mid-gestation (Cloud *et al.*, 2002), this shows that E2F3a can fully substitute for E2F1 and E2F3 genes in the vast majority of murine tissues. This does not assume that E2F3a is acting alone to mediate all of the functions of E2F1, E2F3a and E2F3b, but that the combination of E2F3a and the other endogenous E2F family members gives sufficient total E2F activity to allow near normal development. Given these observations, we conclude that the E2F network has a significant degree of redundancy and robustness, reminiscent of that seen for other core cell cycle regulators including the cyclins and cdks (Berthet and Kaldis, 2007). In contrast to the *E2f1^{-/-};E2f3b^{-/-}* animals, we found that the *E2f1^{-/-};E2f3a^{-/-}* double mutants arise at expected frequency but die within the first few weeks of life. Thus, at least in the context of E2F1 loss, E2F3a plays a more important role than E2F3b *in vivo*. A similar result was observed *in vitro*: *E2f1^{-/-};E2f3a^{-/-}* double mutant MEFs exhibit a reduced level of proliferation, are impaired in their ability to re-enter the cell cycle and fail to appropriately regulate E2F-target genes. In contrast, preliminary analysis suggests that the *E2f1^{-/-};E2f3b^{-/-}* double mutant lines are essentially normal, again indicating a more important role for E2F3a. It is formally possible that E2F3a has one, or more, function(s) that are specifically shared by E2F1 and not other E2Fs. However, we favor the hypothesis that this reflects some difference in the relative levels, or timing of expression, of the two isoforms such that E2F3a, but not E2F3b,

places the total E2F activity above a critical threshold. Notably, our analysis of the *E2f1*;*E2f3a* mutant neonates also reveals an essential role for E2F1 plus E2F3a in cartilage development. Specifically, we find that the *E2f1*^{-/-};*E2f3a*^{-/-} chondrocytes have an abnormal morphology, are not appropriately organized within the epiphysis and are significantly larger than normal. This appears to be an exacerbation of a milder phenotype in *E2f1* mutant embryos. Further experiments will be required to establish the precise cause of these defects. However, our analysis suggests that the chondrocyte phenotype could be due to reduced E2F target gene expression. One intriguing possibility is that these cells have increased their size in preparation for cell division but have trouble proceeding through the cell cycle because there is insufficient accumulation of E2F target gene products, many of which are rate limiting for DNA replication and mitosis.

EXPERIMENTAL PROCEDURES

Generation of mutant mouse strains

A BAC clone (19H05) containing *E2f3* was obtained from a library derived from 129S6/SvEvTac genomic DNA (RPCI-22, Roswell Park Cancer Institute, Buffalo, NY, USA) and transferred into the *E.coli* strain DY380 (Liu *et al.*, 2003) for generation of targeting constructs by recombineering (<http://recombineering.ncifcrf.gov/>). *LoxP* flanked PGKEM7neobpA cassettes were generated from PL452 by PCR (Expand High Fidelity PCR System, Roche, Indianapolis, IN, USA) to contain 50bp of flanking homology to either the first exon of *E2f3a* or that of *E2f3b* and inserted into the BAC

clone *via* recombineering. This insertion resulted in the removal of the two in frame ATGs and the intervening 34 codons from the first exon of *E2f3a* and the sole ATG from exon one of *E2f3b*. Following verification by PCR a 15kb fragment of the BAC encompassing the integrated cassette was transferred into pBR322 (NEB, Ipswich, MA, USA) by gap repair. This clone was transferred into the *E.coli* strain DH5 α and a blunt 8.6kb BstXI – Hpa I fragment transferred to a EcoRV cut pBRDTA01 vector containing a diphtheria toxin negative selection cassette (DTa) using standard cloning procedures (Sambrook *et al.*, 1989). All modifications were verified by restriction digestion mapping and sequencing. The resulting targeting vectors were electroporated into J1 ES cells (derived from 129S4/SvJae) and DNA from 240 (*E2f3a*) and 432 (*E2f3b*) G418-resistant colonies was screened by southern blotting (see below). C57BL/6 blastocysts were injected with correctly targeted ES cells and transplanted into pseudopregnant CD1 mice. Chimeras resulting from one *E2f3a*^{+/-} and two *E2f3b*^{+/-} ES cell lines gave germline transmission, as judged by southern blotting. These were mated to C57BL/6 TgN(ACTB-cre) 2Mrt or 129S4/SvJae Tg(Prm-cre)58Og mice (Jackson Laboratories, Bar Harbor, ME, USA) to delete the *loxP* flanked *neo* cassette, as verified by southern blotting and PCR analysis described below. The *E2f3* mutant alleles were segregated from *Cre* transgenes by breeding to generate *E2f3a*^{+/-} or *E2f3b*^{+/-} strains.

Oligonucleotides (Integrated DNA Technologies) used for generating targeting constructs.

a) Primers to amplify the *loxP* flanked PGKEM7neobpA cassette derived from PL452 (Liu *et al.*, 2003) for integration into the BAC clone by recombineering had the following sequences:

E2f3a

1) AGCAATACGTTAATATATCGTAACACTAAAAAAGAGCAGGAGCGAGAGAT
AACTTCGTATAGCATACA

2) GGGCGGCGGCGGCGGCGGAAGCCGGGGCTGGCTAGCAGTGCCCTTTTGTCA
TAACTTCGTATAATGTATG

E2f3b

1) CTCTCCAGCCGGCCCCCACCTCCCCGGAGCCAGGCTGCTTTCGGAAAT
AACTTCGTATAGCATACA

2) GTGGCTCGGGCCCCCGCGCGGTCCAGGTCACTAACCTGCTGCTGTAAGGG
ATAACTTCGTATAATGTATG

b) Primers used to assess integration of the PGKEM7neobpA cassette:

5' end *E2f3a*

1) GAAAAGAGAGAGAGAGGGTTCGG
2) TCGATATCAAGCTTATAACTCG

3' end *E2f3a*

1) GGTCAGGGCGCCACTTTGGAGG
2) GCCGCATAACTTCGTATAGC

5' end *E2f3b*

1) GGACGGTCCCGGCGCCCTCGCACC
2) CTTCCATTTGTCACGTCCTGC

3' end *E2f3b*

1) GGTCTTAATTAAGTCTGGGCCAAGGCACAACACGAGG
2) GCCGCATAACTTCGTATAGC

c) Primers for gap repair retrieval of 15kb fragments from targeted BAC into pBR322:

1) GCCAATTGGATGTCTATTTTGAGAAAGAGAGGCCAGTTAGATTGTTCCAG
GACGAAAGGGCCTCGTGATACGCC

2) TCGAGTCTTGCTTACACTACCATCACGTCTGTCCTTCCGATTTCAGCCTC
CCGATACGCGAGCGAACGTGAAGC

d) Local sequence following removal of the *neo* cassette. ATGs shown in upper case, *loxP* sequence in italics and stop codons underlined. In both cases the *loxP* site contributed an in-frame stop codon.

E2f3a:

Codon number: 1 34

Wild-type: gagcgagagATG aga aag//ATG gac aaa

Mutant: gagcgagagataaacttcgtatagcatacattatacgaagtatgac aaa

E2f3b:

Codon number: 1

Wild-type: ctctcgaaATG ccc tta cag cag cag

Mutant: ctctcgaaataaacttcgtatagcatacattatacgaagtatccc tta cag cag cag

Southern Blotting analysis of targeted ES cells.

ES cell DNA was isolated from targeted clones as described (Laird *et al.*, 1991) and digested with BglIII for analyses using a 676bp 5' probe (*E2f3a* and *E2f3b*) and either SphI (*E2f3a*) or HindIII (*E2f3b*) for analyses using a 911bp 3' probe by Southern blotting. A probe hybridizing to the *neo* cassette was also used to identify clones containing a single insertion of the targeting construct be re-probing the 5' probe blots following stripping of the membrane. Southern blotting was performed using Hybond N

membranes (Amersham Biosciences, Piscataway, NJ, USA), ExpressHyb (Clontech, Mountain View, CA USA) and standard procedures (Sambrook *et al.*, 1989).

Radiolabeled probes were generated using $\alpha^{32}\text{-P}$ dCTP (Perkin Elmer, Waltham, MA, USA), a Prime-It II kit (Stratagene, La Jolla, CA, USA) and purified using QuickSpin TE columns (Roche). Five of the 240 *E2f3a* clones and eight of the 432 *E2f3b* clones were judged to be correctly targeted.

Primers used to generate probe fragments for Southern blotting using the BAC clone 19H05 or PL452 as a template:

5' probe

1) ATCCTGCAGCTGATCAATGAGCAGGCTGGGG

2) ATCCTGCAGACTTGTTGCCAAGAGCTACACC

3' probe

1) ATCCTGCAGTGACTGGCTGTAAGCATTGTCC

2) ATCCTGCAGTACGCTAACCTGAAATACTGTCC

Neo cassette

1) GGTCGCTAGCCGGATCGGCCATTGAACAAGATGG

2) CCTCGCTAGCTGATCACTCAGAAGAACTCGTCAAGAAGGCG

Genotyping protocols for *E2f3a* and *E2f3b* mutant alleles.

Pfu polymerase (Stratagene) buffer containing 1% (*E2f3a*) or 5% (*E2f3b*) DMSO and 250nM oligonucleotides. *E2f3a*: Primers GGTCAGGGCGCCACTTTGGAGG and AGCAATACGTTAATATATCGTAACAC (Integrated DNA Technologies, Coralville, IA, USA). PCR protocol: 94°C 2min; 94 °C 30s, 58 °C 30s, 72 °C 1min x34 cycles, 72°C 7min. Product sizes: wild-type allele 276bp, mutant allele 210bp. *E2f3b*: Primers

CCCGGAGCCAGGCTGCTTTCGG and CCCC GTTCCCACTCCAAAACC. PCR protocol: 94°C 2min; 94°C 30s, 62°C 30s, 72°C 70s x30 cycles, 72°C 5min. Product sizes: wild-type allele 157bp, mutant allele 188bp. *E2f1* genotyping was performed as described (Cloud *et al.*, 2002). The χ^2 test was used to test the null hypothesis that the frequency of test genotypes did not deviate significantly from the predicted frequency.

Genotyping protocols for Cre recombinase expressing alleles and the *loxP* flanked *neo* cassette prior to excision.

PCR was used to detect the presence of transgenes containing *Cre*.

Oligonucleotide primers were used at a final concentration of 250nM:

GAAAACGTTGATGCCGGTGAACG and GTAACAGGGTGTTATAAGCAATCC, expected product 150bp.

PCR protocol: 94°C 2min; 94 °C 30s, 55 °C 30s, 72 °C 1min x34 cycles, 72 °C 4min.

PCR buffer (x10): 500mM KCL, 100mM Tris pH8.5, 10mM MgCl₂.

Primers used to detect the presence of the *neo* cassette in transgenic mice. PCR conditions described above.

E2f3a allele: AGCAATACGTTAATATATCGTAACAC and CTTCCATTTGTCACGTCCTGC, expected product 338bp.

E2f3b allele: CCCC GTTCCCACTCCAAAACC and GAGGATTGGGAAGACAATAGC, expected product 286bp.

MEF generation and analyses

Passage 4 MEF lines were prepared as described (Humbert *et al.*, 2000). Mutant MEFs were always compared with littermate controls. Proliferation assays and cell cycle re-entry experiments were performed essentially as described (Aslanian *et al.*, 2004)

apart from the determination of S-phase progression which was monitored by FACS based detection of incorporated BrdU using a FITC conjugated anti-BrdU antibody (347583 BD Biosciences, San Jose, CA, USA) as described (Janumyan *et al.*, 2003), using a BD FACScan. For Western blotting, protein extracts were prepared in 20mM Tris pH7.5, 250mM NaCl, 5mM NaF, 1mM EDTA, 1mM EGTA, 1% Triton as described using antibodies against E2f3, LLF3#2G2 (Parisi *et al.*, 2007), p19Arf (sc32748, Santa Cruz Biotechnology, Santa Cruz, USA), p21^{Cip1} (sc6246, Santa Cruz Biotechnology) and GAPDH (4300, Ambion, Austin, TX, USA). ChIP was performed as described (Aslanian *et al.*, 2004). For quantitative PCR, RNA was collected from cells at 0, 12, 16, 20, and 24 hours after re-entry and RNA was processed as described (Courel *et al.*, 2008).

Primers used for qPCR analysis:

5'-CACCGGAATCCTGGACCAG-3' and 5'-GCAGTTCGAATCTGCACCGT-3' for *Arf*, 5'-GATGCTCATCTGACCGGAGT-3' and 5'-ATAAGTCACGTAGGCGCACA-3' for *p107*, 5'-GACAAGAGGCCCACTTCC-3' and 5'-CAATCTGCGCTTGGAGTGATA-3' for *p21*, 5'-AGTTTGATAGATGCTGACCC-3' and 5'-TAGGTCTGGTGAAGGTCC-3' for *Cyclin A*, 5'-TGTTTTTGCAAGACCCAGATGA-3' and 5'-GGCTGACTGCTATCCTCGCT-3' for *Cyclin E*.

Histological analyses and immunohistochemistry

Soft tissues were fixed in 3.7% formaldehyde in PBS overnight whilst adult bones were fixed in Bouin's fixative (Poly Scientific, Bat Shore, NY, USA) for 10 days. Paraffin sections were cut at 5 μ m, dewaxed and stained with hematoxylin and eosin. To assess proliferation, femur sections from a minimum of three pairs of control and mutant

littermates were matched along the proximal-distal axis and immunohistochemistry was performed using antibodies raised against Ki67 and BrdU following BrdU labeling as described (Danielian *et al.*, 2007). The percentage of positive nuclei was determined by counting 150-1000 nuclei within each zone in the femoral cartilage per section and the results analyzed by Student's t-Test. Cell area measurements were made using ImageJ software. A minimum of 35 cells of each chondrocyte type from each femoral head were measured and subject to the Student's t-Test and box-plot analyses. In all quantification studies at 18.5dpc between three and six pairs of control and mutant littermates embryos or pups were analyzed and both femurs were sectioned.

Immunohistochemistry for Collagen X and Collagen II.

For all procedures paraffin sections on slides were re-hydrated through an ethanol series following de-waxing in xylenes and rinsed in water or PBS (phosphate buffered saline) as required.

Collagen II: slides were washed in PBS 0.15% Triton X-100 followed by inactivation of endogenous peroxidases by incubation with 3% H₂O₂ in PBS. Antigen retrieval was performed by heating for 20 minutes in 10mM sodium citrate, 0.05% Tween 20, pH6.0 in a boiling water bath. Slides were blocked with PBS containing 5% rabbit serum and incubated overnight at 4°C with primary antibody (sc-7764, N-19, Santa Cruz Biotechnology) 1/500 in PBS 5% rabbit serum or this buffer alone or a non-specific antiserum as controls. Secondary antibodies (Vectastain Elite ABC kits, Vector laboratories) were diluted 1:200 in PBS containing 0.4% of the appropriate blocking serum and detected using a DAB substrate following the manufacturers instructions

(Vector Laboratories). Following the detection reaction slides were counterstained with Harris hematoxylin and mounted using standard protocols.

Collagen X: Essentially identical to above apart from the following:

Antigen retrieval used a 0.1% pepsin, 0.5M glacial acetic acid solution for 2hrs at 37°C.

Sections were washed in PBS, blocked for 2 hours in 5% horse serum in PBS

and incubated with primary antibody ColX53 (#2031501005, Quartett, Berlin, Germany)

1:20 in PBS 5% horse serum overnight at 4°C. The peroxidase step was performed after the primary antibody incubation. The remainder of the protocol is as described above.

For each marker analyzed a minimum of three pairs of control and mutant littermate matched sections were stained and, unless stated otherwise, all scored with the described phenotype. Images were captured on a Nikon Eclipse E600 using a SPOT RTdigital camera.

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Supplementary Figure 1

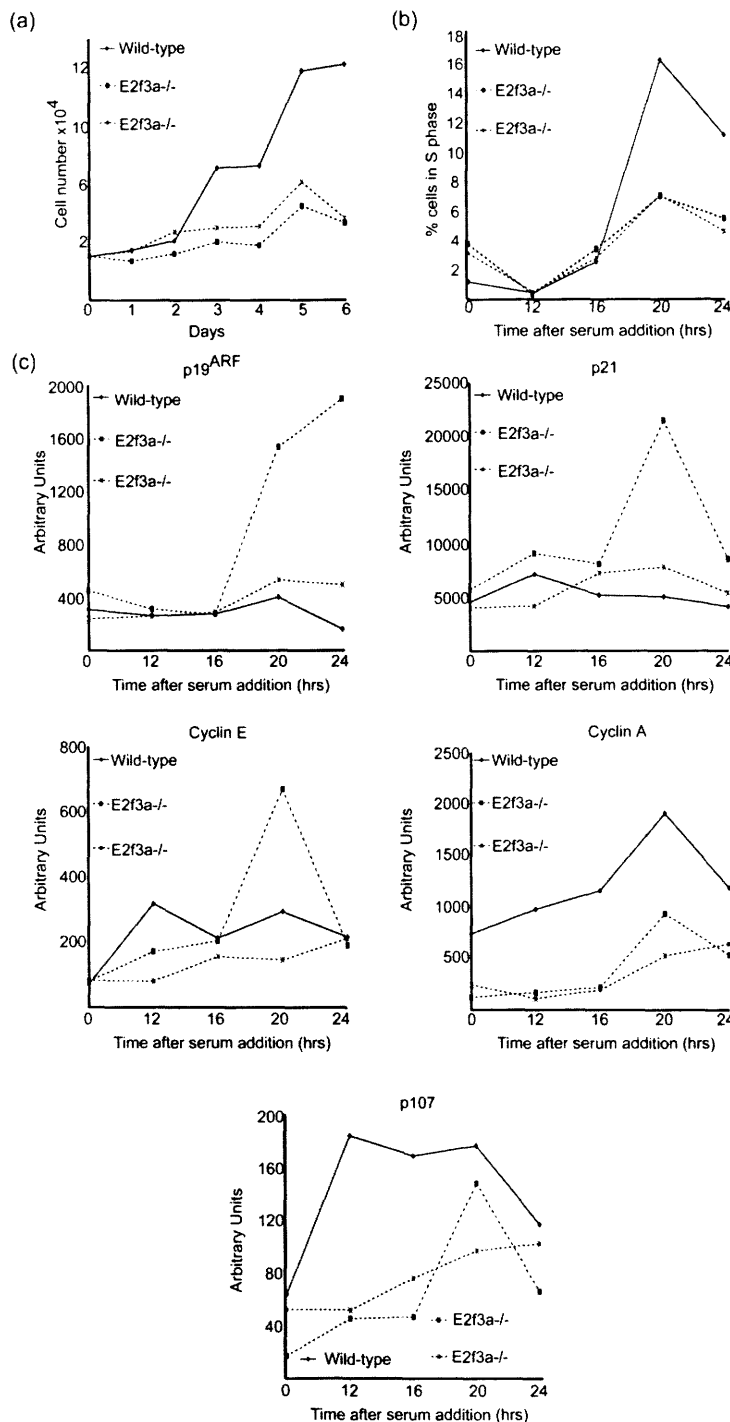


Figure 1: Proliferation and cell cycle re-entry defects occur in some *E2f3a* mutant MEF lines.

(a) *E2f3a*^{-/-} and wildtype littermate MEFs were assayed for asynchronous proliferation. Cells were plated in duplicate at 6x10⁴/3cm dish and their growth was monitored by daily counting for six days. (b) Wild-type and *E2f3a*^{-/-} MEFs were serum starved (T₀) and induced to enter into the cell cycle with serum. Entry into the cell cycle was analyzed by BrdU incorporation at the indicated times (hours) followed by propidium iodide staining and FACS analysis, the percentage of cells in S-phase (BrdU labeled) is plotted. (c) Quantitative PCR analyses of the mRNA expression levels of *Arf* and *p21* as well as the E2f target genes *Cyclin E*, *Cyclin A* and *p107* in *E2f3a*^{-/-} and wild-type littermate MEFs during the cell cycle re-entry experiment.

Supplementary Figure 2

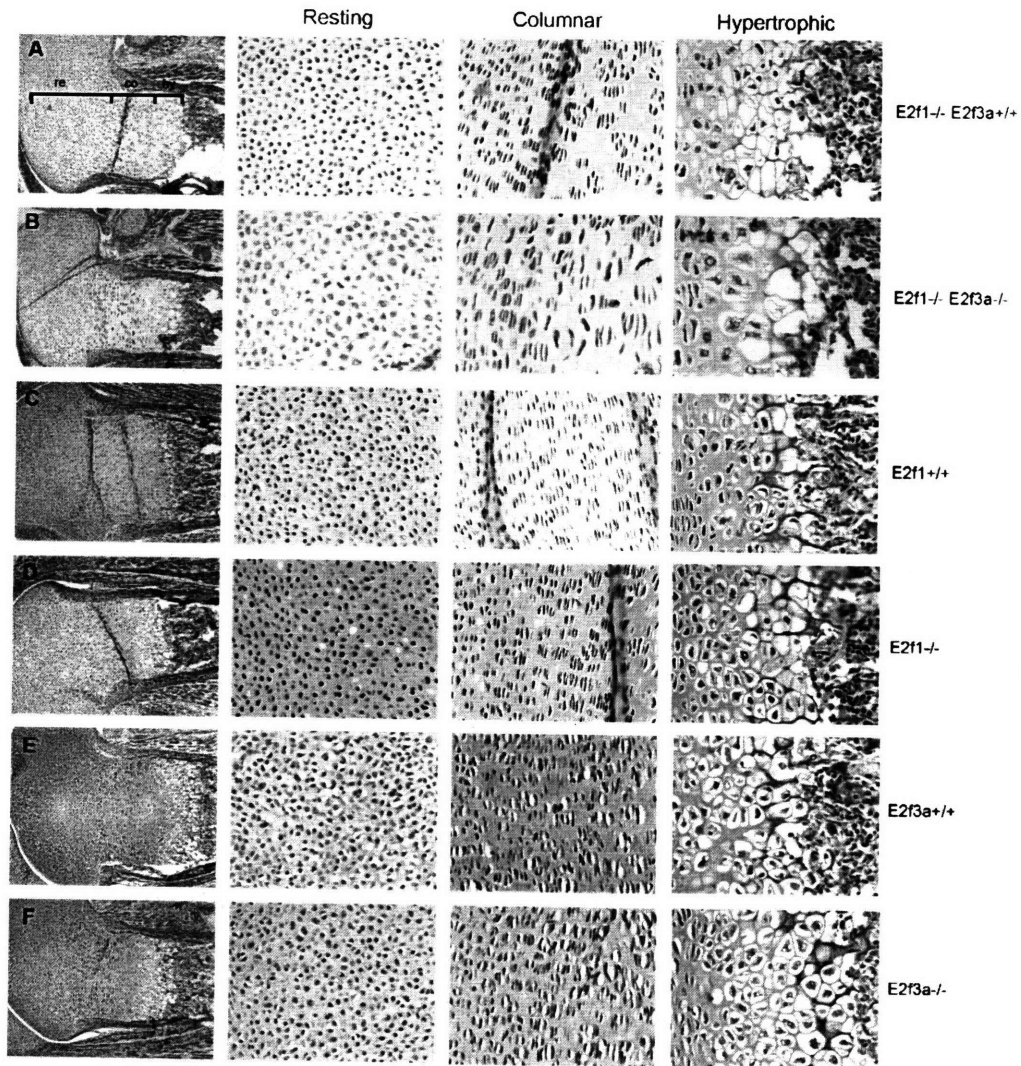


Figure 2: Histological analyses of femoral cartilage at 18.5dpc in *E2f1;E2f3a*, *E2f1* and *E2f3a* mutant embryos.

Representative sections of the indicated control littermate (A, C and E) and mutant embryo femurs (B, D and F) are shown. The first panel in each row x10, remaining panels x40. Chondrocytes in *E2f1;E2f3a* mutants are substantially larger than normal chondrocytes. Resting, re; columnar, co; hypertrophic hy.

Supplementary Figure 3

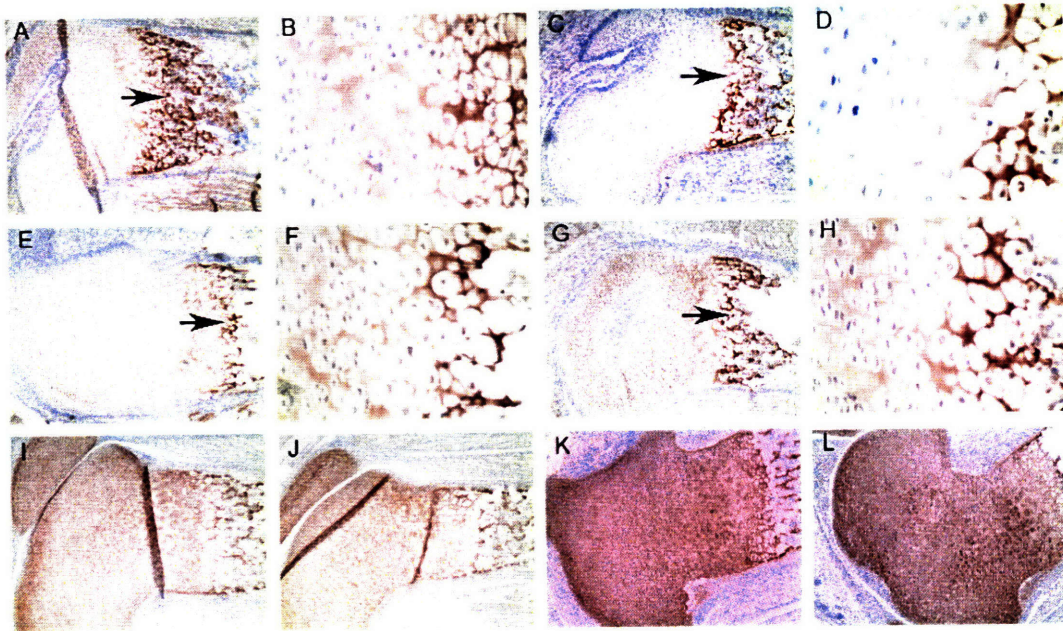


Figure 3: Collagen X and Collagen II are expressed normally in *E2f* mutant femurs. Collagen X staining in (A, B) *E2f1*^{-/-};*E2f3a*^{+/+} (C, D) *E2f1*^{-/-};*E2f3a*^{-/-} (E, F) *E2f1*^{+/+} and (G, H) *E2f1*^{-/-} samples. In all cases Collagen X staining is restricted to hypertrophic chondrocytes (arrows) and no ectopic expression in other types of chondrocytes is observed. Collagen II expression in (I) *E2f1*^{-/-};*E2f3a*^{+/+} (J) *E2f1*^{-/-};*E2f3a*^{-/-} (K) *E2f1*^{+/+} and (L) *E2f1*^{-/-} samples was detected throughout the cartilage template in all genotypes. Panels B, D, F and H x40, remaining panels x10.

Appendix B

A role for RYBP in chromatin condensation

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INTRODUCTION

The polycomb group (PcG) proteins form large multimeric complexes that influence the structure and transcriptional state of chromatin (Jacobs and van Lohuizen, 2002). PcG proteins exist in at least two distinct core polycomb repressive complexes (PRCs) (Saurin *et al.*, 2001; Kuzmichev *et al.*, 2002; Levine *et al.*, 2002). PRC2 consists of the proteins EED, EZH, and Suz12 (Cao *et al.*, 2002; Kuzmichev *et al.*, 2002; Muller *et al.*, 2002). This complex initiates a heritable, repressive state of chromatin through physical modifications to the tails of histones. Specifically, EZH2 methylates histone H3 at lysine 27 (Cao *et al.*, 2002; Czermin *et al.*, 2002; Kuzmichev *et al.*, 2002; Muller *et al.*, 2002). The PRC2 complex is necessary for targeting the PRC1 complex to genomic loci (Cao *et al.*, 2002). The PRC1 core complex contains the proteins Bmi1, Ring1a, Ring1b, HPH, HPH2, HPC2, HPC3, and Scmh1 (Saurin *et al.*, 2001; Levine *et al.*, 2002). Most importantly, HPC, a homologue of the *Drosophila* polycomb (Pc) protein, directly binds to methylated histone H3 at lysine 27 through a highly conserved chromodomain (Cao *et al.*, 2002; Muller *et al.*, 2002). Recently, it has been found that Ring1a and Ring1b have catalytic E3 ubiquitin ligase activity towards histone H2A at lysine 119. Ring1b maintains global H2A ubiquitination in ES cells, and both Ring1a and Ring1b can ubiquitinate histone H2A on the inactive-X chromosome (de Napoles *et al.*, 2004; Cao *et al.*, 2005). The big question is whether the ubiquitin chromatin mark contributes to repression or whether it has another function. To answer this question, Cao *et al.* found evidence that this chromatin mark participated in repression by PRCs and that H2AK119 ubiquitination is downstream of histone H3 lysine 27 methylation (Cao *et al.*, 2005).

Similar to the Pc protein, RYBP (Ring1 and YY1 Binding Protein) binds directly to the ubiquitin chromatin mark *in vivo* and co-localizes with Ring1a at the inactive-X chromosome (Arrigoni *et al.*, 2006).

RYBP interacts with YY1, E2Fs, and core PRC1 components (Garcia et al., 1999). Results from this study indicate that RYBP plays a vital role in the formation of condensed chromatin. Additionally, there is evidence to suggest that RYBP interacts with Suv420, a histone methyl transferase (HMTase) that methylates histone H4 at lysine 20. The results implicate this histone methyl mark in PcG-mediated silencing and present a vital role for RYBP in chromatin condensation.

RESULTS

Overexpression of RYBP in U-2 OS cells leads to chromatin condensation.

When expressed at low levels in a human osteosarcoma cell line (U-2 OS), RYBP co-localizes with Bmi1 (Figure 1A) in repressive nuclear domains termed polycomb bodies. Endogenous E2F6, although mostly diffuse in the nucleus, also localizes to punctate nuclear foci, suggesting that E2F6 may be present in polycomb bodies. (Figure 1B). When RYBP is overexpressed in human U-2 OS cells, genome-wide condensation is observed (Figure 2A). These condensed chromatin “spots” have features of heterochromatin, including histone H3 lysine 9 methylation and an absence of histone H3 acetylation (Figure 2B). These findings indicate that RYBP overexpression is causing genome-wide reorganization of the DNA into heterochromatin.

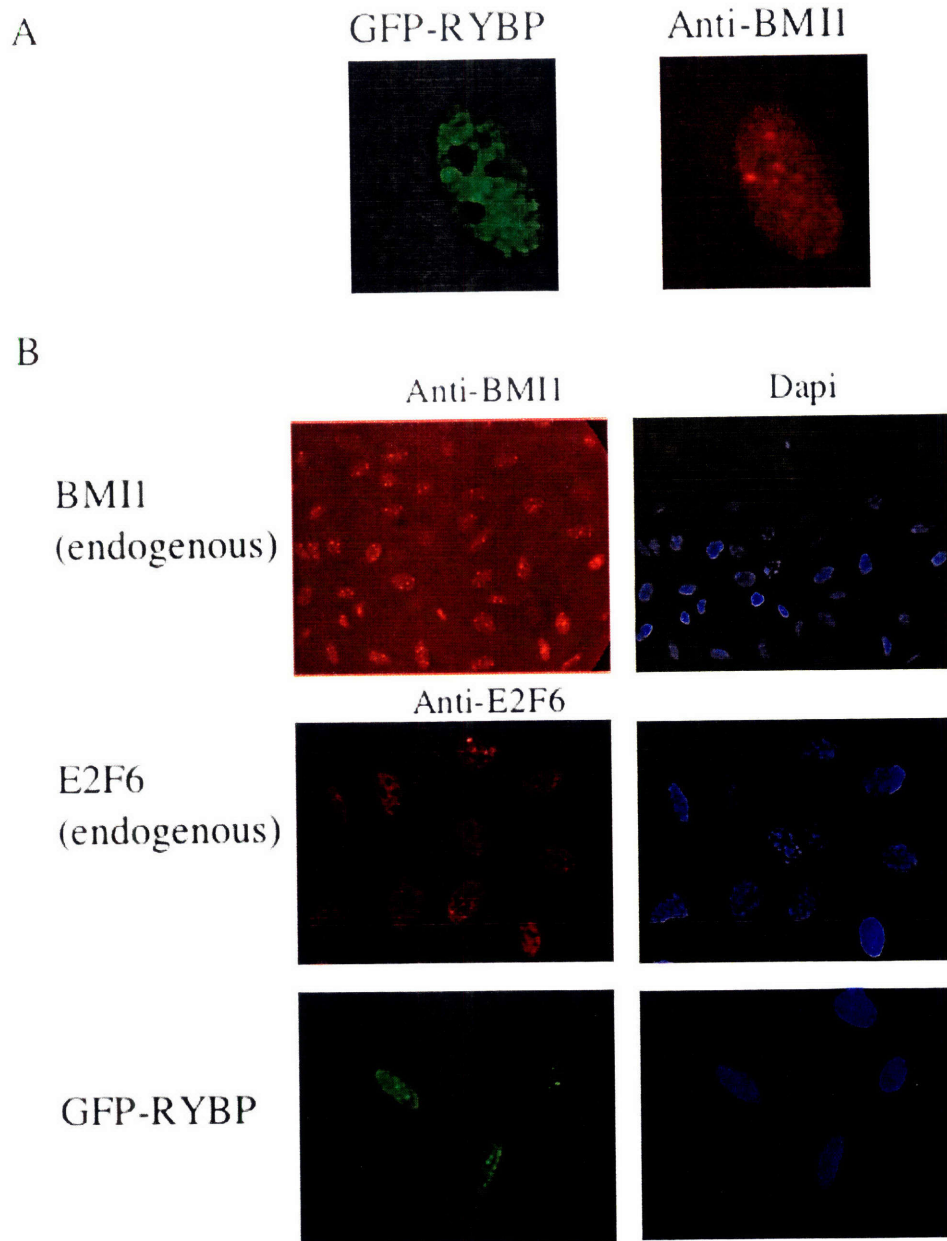


Figure 1: RYBP, Bmi1 and E2F6 localize to polycomb bodies.

(A) RYBP expressed at low levels co-localizes with Bmi1 in polycomb bodies. (B) RYBP, Bmi1, and E2F6 all have a punctate nuclear expression pattern.

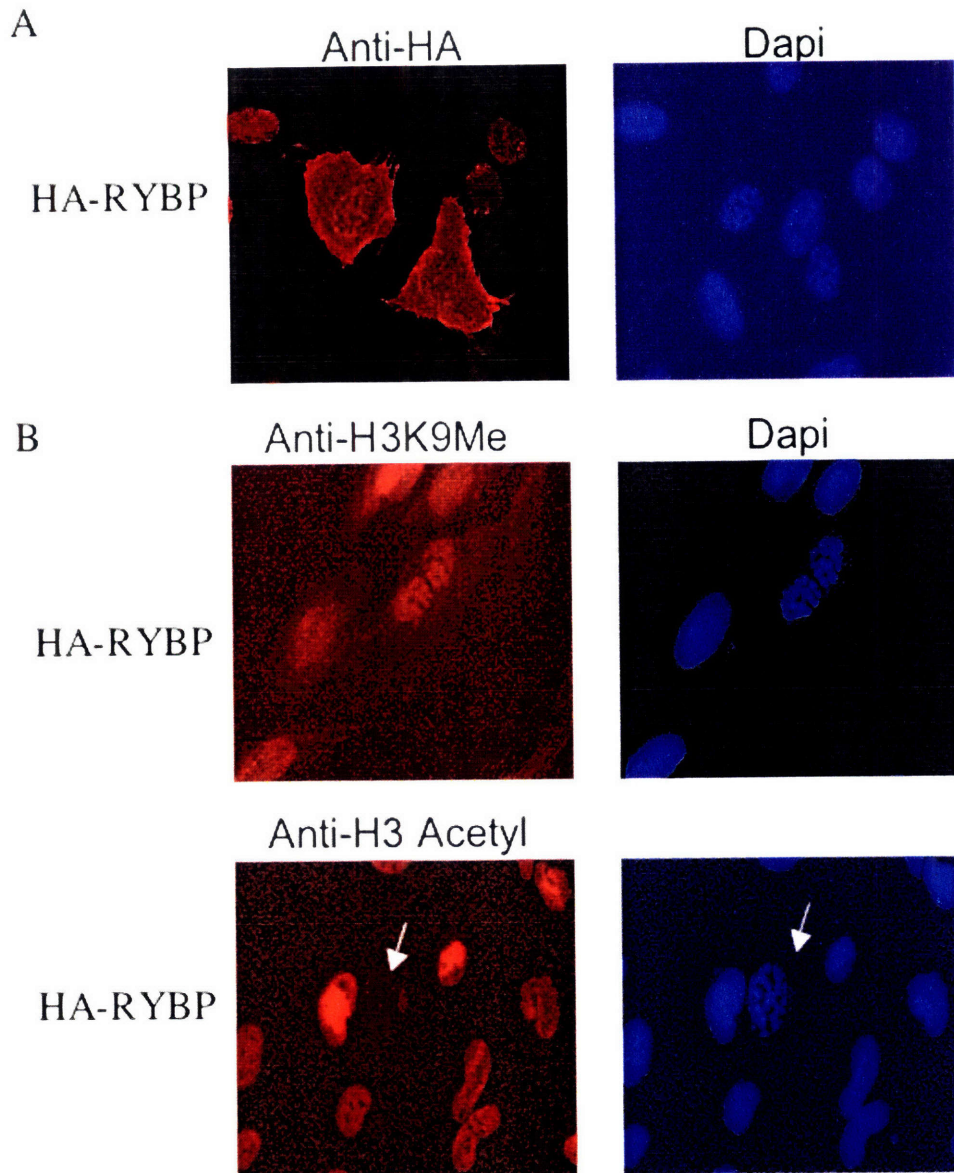


Figure 2: RYBP overexpression results in the formation of heterochromatin.
 (A) RYBP overexpression in U-2 OS cells results in the formation of condensed chromatin Dapi “spots.” (B) The condensed chromatin have marks of heterochromatin including H3K9 methylation and a loss of H3 acetylation.

RYBP and Cellular Senescence

In addition to the heterochromatic changes to the DNA, the U-2 OS cells overexpressing RYBP exhibit a senescent-like phenotype. Cellular senescence has classically been defined as an irreversible growth arrest of cultured cells. Although the cells remain metabolically active, they no longer respond to growth stimuli. Senescent cells display characteristic changes in cell morphology, gene expression, and an up-regulation of senescence-associated β -galactosidase activity (Dimri *et al.*, 1995). The products of the *Ink4a-Arf* locus, p16^{INK4a} and p19^{Arf}, are important senescent regulators, and they exert their effects through regulation of p53 and pRB. p16^{INK4a} inhibits cyclin D phosphorylation of pRB while p19^{Arf} promotes stabilization of p53 by inhibiting Mdm2 (Lundberg *et al.*, 2000). Both p16^{INK4a} and p19^{Arf} accumulate in senescent cells, and it is believed that pRB is required for cellular senescence (Narita *et al.*, 2003). A variety of polycomb proteins have been shown to induce cellular senescence (Jacobs *et al.*, 1999b; Gil *et al.*, 2004). In each case, the actions of these proteins require the presence of p53 and pRB (Itahana *et al.*, 2003).

To test the requirement of p53 and pRB, we overexpressed RYBP in a human transformed cell line, C33, which is deficient for both p53 and pRB. Overexpression of RYBP induces chromatin condensation in C33 cells (Figure 3A), suggesting that RYBP has a more direct role in the chromatin condensation process than any of the previously characterized regulators. When primary human fibroblast (IMR90) cells are depleted of RYBP by shRNA knockdown, these cells also exhibit a senescent-like morphology (Figure 3B) and an increase in senescence-associated β -galactosidase activity (Figure

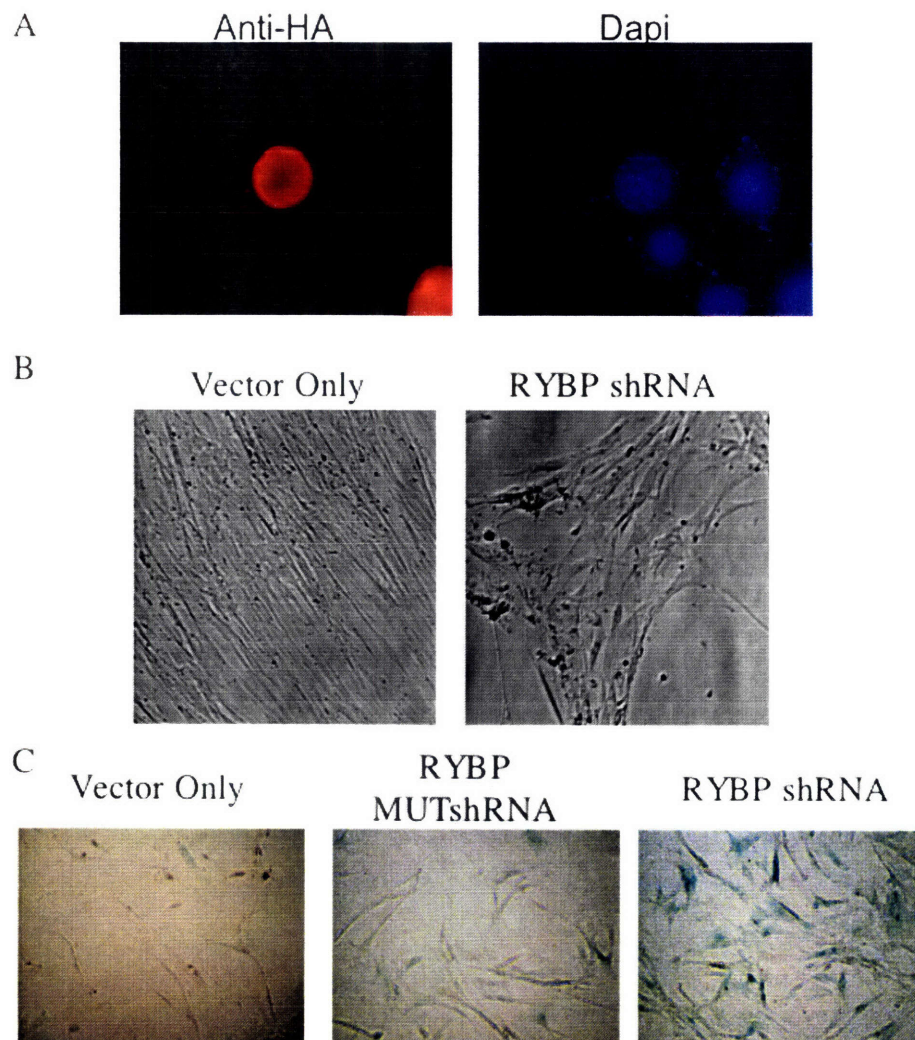


Figure 3: RYBP chromatin condensation is independent of p53 and pRB, and knockdown of RYBP results in cellular senescence.

(A) RYBP overexpression in cells deficient for pRB and p53 results in condensed chromatin DAPI "spots." (B) IMR90 cells expressing a shRNA to RYBP have a cellular morphology indicative of senescence, and (C) increased senescence-associated β -galactosidase activity.

3C). No increase in senescence-associated β -galactosidase activity is seen when the vector alone or a point mutant shRNA is expressed in cells (Figure 3C). One hypothesis to explain these results is that RYBP is part of the core machinery responsible for the repression of chromatin. Overexpression of RYBP may cause heterochromatic formation non-specifically over the entire genome, and knockdown of RYBP may cause senescence due to the cell's inability to properly repress chromatin.

E2F6's requirement in chromatin condensation

To further investigate RYBP dependent chromatin condensation, we used immunofluorescence to identify other proteins that are involved in this process. Interestingly, co-expression of DP1, the E2F dimerization partner, with RYBP suppresses the chromatin condensation (Figure 4A). These findings implicate E2F in this process. One hypothesis is that overexpression of DP1 titrates an E2F from the RYBP complex. The most obvious candidate E2F to participate in this process is E2F6, since E2F6 interacts with RYBP and other PcG proteins (Trimarchi *et al.*, 2001). Thus, to examine E2F6's role in chromatin condensation, we used immunofluorescence to determine the localization of E2F6. Endogenous (Figure 4B) and overexpressed (Figure 4C) E2F6 localizes to the condensed heterochromatin. There are two hypotheses to explain the localization of E2F6 to the condensed chromatin: (1) E2F6 specifically participates in the process of chromatin condensation or (2) the DNA binding activity of E2F6 localizes it to the chromatin, but it plays no direct role in chromatin condensation. Since GFP-E2F4 does not localize to the condensed chromatin (data not shown), the second hypothesis is

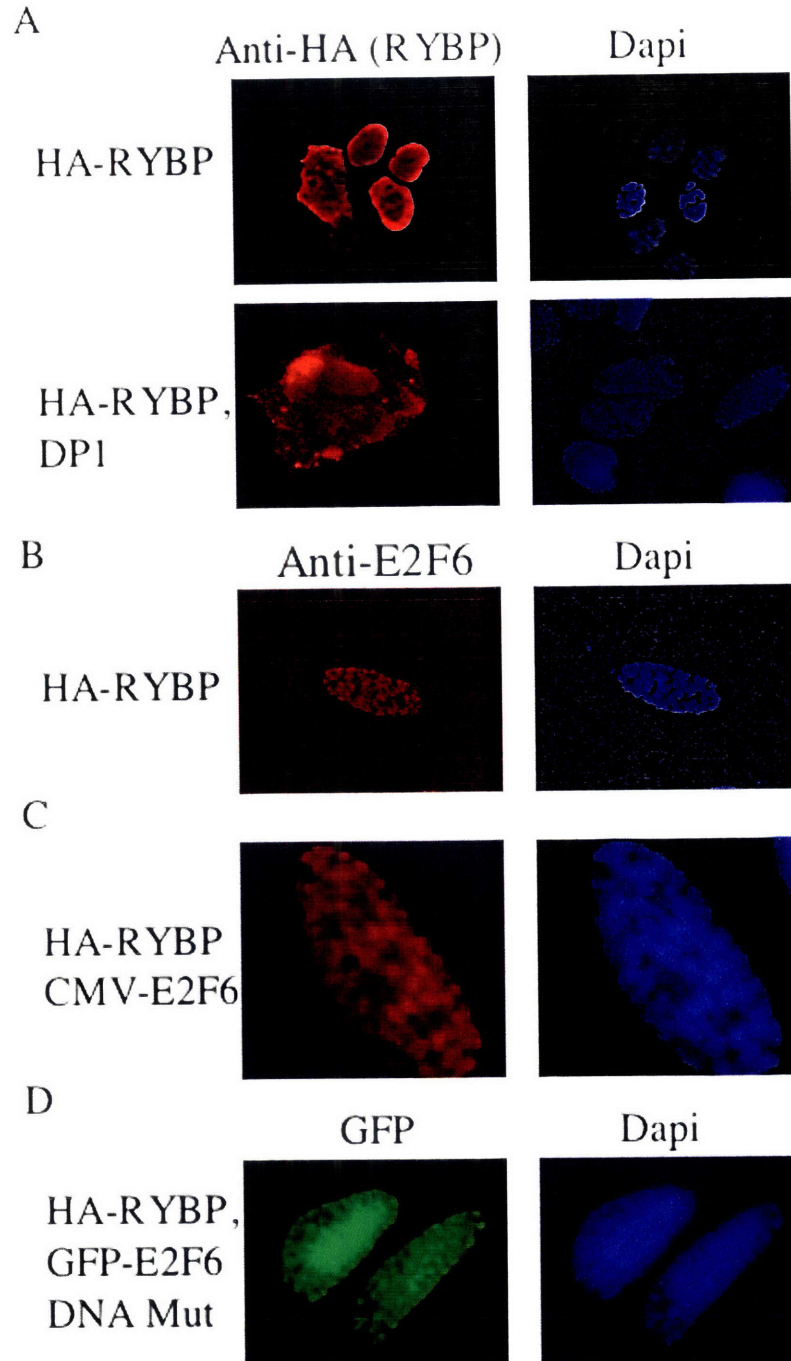


Figure 4: E2F involvement in chromatin condensation.

(A) RYBP and DP1 co-expression suppresses the chromatin condensation. (B) Endogenous and (C) overexpressed E2F6 localizes to the condensed chromatin. (D) A GFP-E2F6 DNA binding mutant localizes to the condensed chromatin.

less likely to be correct. These results imply that E2F6 plays a direct role in RYBP-mediated chromatin condensation. One possibility is that the DNA binding activity of E2F6 recruits RYBP to the DNA. However, an E2F6 DNA binding mutant still localizes to the condensed chromatin (Figure 4D).

Interaction of RYBP and Suv420

Suv420 methylates histone H4 at lysine 20 and acts downstream of histone H3 lysine 9 and histone H3 lysine 27 methylation (Schotta *et al.*, 2004). This HMTase was identified as an interactor of RYBP in a yeast two-hybrid screen (F Connor, JA Lees, unpublished data). GFP-Suv420 expressed in U-2 OS cells co-localizes with RYBP in polycomb bodies (Figure 5A). Overexpression of GFP-Suv420 results in a chromatin condensation phenotype similar to that seen in RYBP overexpression (Figure 5B). These results strongly suggest that RYBP interacts with Suv420, and that H4 lysine 20 methylation is involved in RYBP-mediated chromatin condensation.

DISCUSSION

Polycomb group proteins are known regulators of chromatin structure. Some PcG proteins directly modify chromatin while others bind directly to chromatin marks. Recently, it has been demonstrated that PRC1 can ubiquitinate histone H2A. Specifically, Ring1a and Ring1b exhibit E3 ubiquitin ligase activity towards histone H2A (de Napoles *et al.*, 2004; Wang *et al.*, 2004; Cao *et al.*, 2005). Arrigoni *et al.* discovered that RYBP, another PcG protein, binds directly to ubiquitinated H2A *in vivo* (Arrigoni *et al.*, 2006).

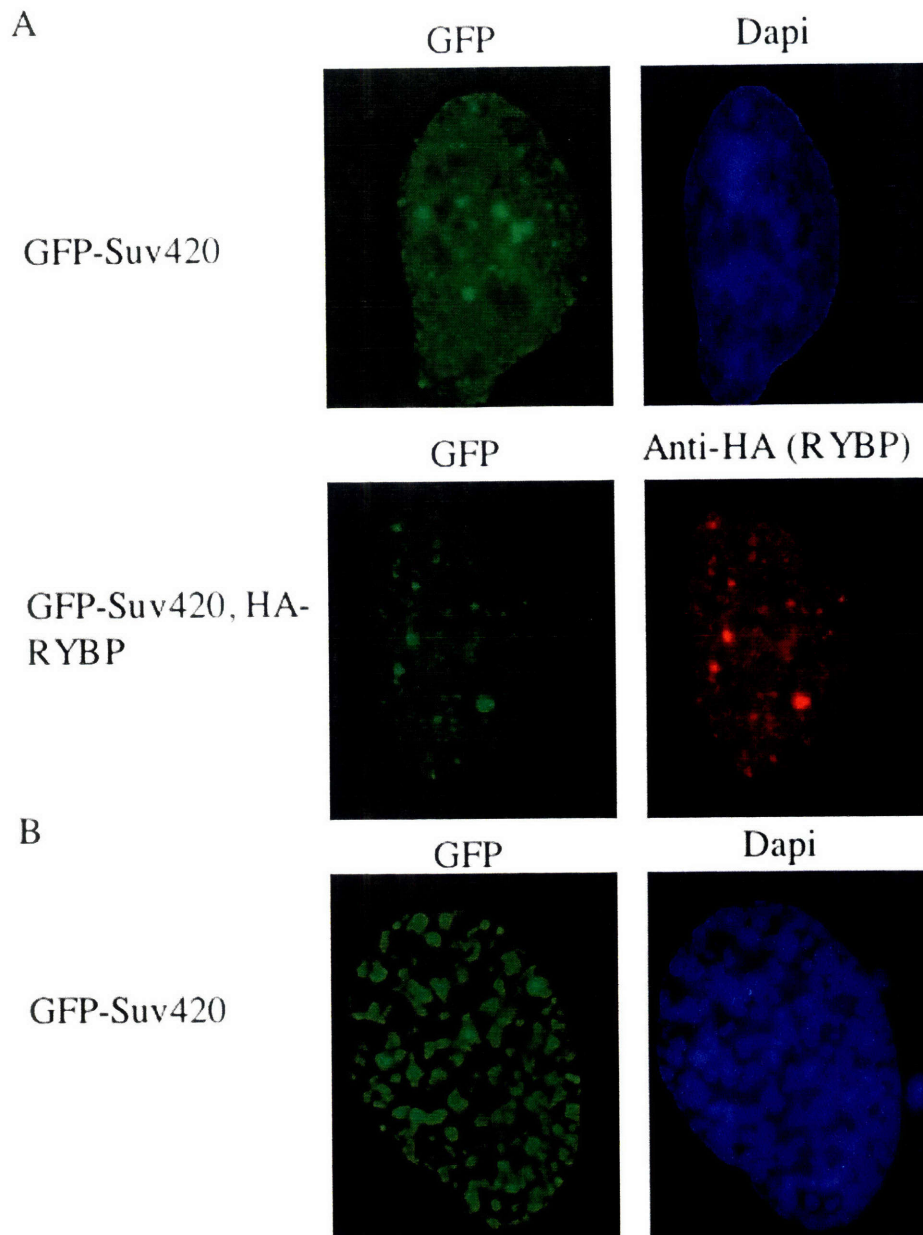


Figure 5: RYBP and Suv420 co-localize to polycomb bodies, and GFP-Suv420 overexpression causes chromatin condensation.
 (A) GFP-Suv420 has a punctate expression pattern (first row) and co-localizes with HA-RYBP (second row). (B) GFP-Suv420 expressed at high levels induces chromatin condensation independently of RYBP overexpression.

The mechanism of how the concerted action of H3K27 methylation and H2AK119 ubiquitination serves to repress chromatin is still unknown.

This study reports a novel role for RYBP in the facilitation of histone H4 lysine 20 methylation. Schotta *et al.* first reported H4K20 methylation to be downstream of histone H3 lysine 9 and histone H3 lysine 27 methylation (Schotta *et al.*, 2004). The results presented here propose an alternate mechanism in which H2A ubiquitination and RYBP recruits a Suv420-containing complex to PcG targets (Figure 6). This methyl mark could lead to a very condensed state of chromatin. It is clear that a “histone code” plays a vital role in the regulation of chromatin. PcG complexes are at the core of this regulation, and their roles in regulation of transcription reach far beyond *Hox* genes.

ACKNOWLEDGEMENTS

Jeff Trimarch made the initial observation of chromatin condensation in U-2 OS cells by RYBP overexpression. Francis Connor performed the yeast two-hybrid screen with RYBP was performed by Francis Connor.

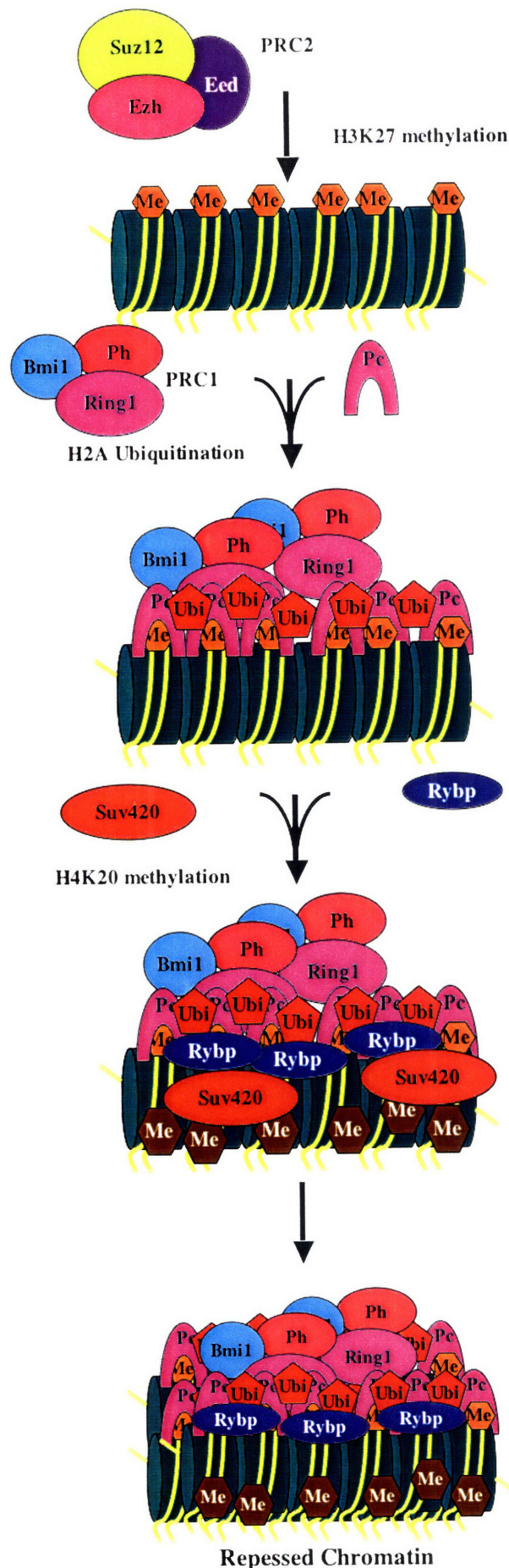


Figure 6: Hypothetical mechanism of PcG Repression of Chromatin

PcG repression is initiated by PRC2 and H3K27 methylation. This methyl mark recruits the polycomb protein and PRC1. Ring1 of PRC1 ubiquitinates histone H2A. RYBP binds directly to ubiquitinated histones. Unpublished data suggests that RYBP associates with a H4K20 methylase, Suv420. This histone methyl mark may participate in PcG repression and lead to repressed chromatin.

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